A STUDY OF THE PHOTOTOXICITY INDUCED IN RATS BY PYROPHEOPHORBIDE *a* AND RELATED DERIVATIVES OF CHLOROPHYLL

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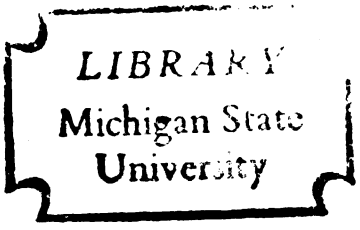

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ABSTRACT

A STUDY OF THE PHOTOTOXICITY INDUCED IN RATS BY PYROPHEOPHORBIDE *a* AND RELATED DERIVATIVES OF CHLOROPHYLL

By Mark H. Love

A severe chorioretinitis, consistently causing complete destruction of the retina, was produced in seventy-five gram albino rats of both sexes when sensitized 24 hours after three milligram intravenous injections of primary photosensitizer, pyropheophorbide *a*. The usual clinical and pathological manifestations of photosensitization also developed, and 38% of the rats developed cataracts. Photosensitivity and intraocular lesions did not develop in rats given only the vehicle and exposed to light. Rats given pyropheophorbide *a*, but held under diffuse light in the animal room, did not have lesions. Pheophorbide *a* produced similar results.

A bioassay system was developed using albino rats, intravenous injection of the suspected photodynamic agent, and exposure under light from cool white fluorescent lamps. The system was used to screen three derivatives of chlorophyll *a*, one of chlorophyll *b*, and the products obtained from the extraction of pork and beef livers for phototoxic agents.

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By

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INTRODUCTION

Diseases caused by light encompass a large number of human and animal afflictions. The occurrence of these in both man and animal have been reported for centuries (Smith and Jones, 96). These diseases are the result of photobiological processes involving the absorption of a quantum of radiation by a substance, the photodynamic agent, in the living system. The characteristic symptoms associated with the syndrome include photophobia, subcutaneous edema, pruritus, erythema of non-pigmented skin, serum leakage, necrosis and skin slough (in later phases), and death during the acute phases.

The chemical nature of the absorbing substance has a direct relation to the type of lesions produced. The type of energy absorbed indicates the chemical structure of the sensitizing substance and is related to the etiology of the disease. Some of the substances absorb energy in the ultraviolet range (290-320 nm) and produce symptoms characteristic of sunburn. The substances which produce the symptoms described above absorb energy of longer wavelengths (longwave ultraviolet 320-400 nm and visible 400-750 nm). Included in this class of chemical substances are the porphyrins; dyes, such as methylene blue and eosin; furocoumarins; sulfanilamides; and rose bengal (Blum, 15).

Diseases caused by photobiological responses have been the subject of many studies. Included in these studies have been the role of the porphyrins (animal, derived from protoporphyrins and hemoglobin, and plant, derived from chlorophyll). Anderson was the first to

suggest the association of porphyrins with the abnormal sensitivity of humans to light, porphyria (Blum, 15). His concern and that of other researchers was the etiology of the disease in humans.

Paralleling the study of human porphyrin diseases were the investigations in South Africa, Australia, New Zealand, and the United States of America implicating plant porphyrins as the causative agent in *geeldikkop* and related diseases in sheep, cattle, horses, and swine (Smith and Jones, 96). The studies of Clare in New Zealand led to his classification of photodynamic agents into two types. The primary agents are pigments and substances (dyes) not usually encountered in the diet and not efficiently excreted or detoxified by the liver. Secondary agents or hepatogenous photodynamic agents produce their phototoxic effects due to a congenital error in metabolism or a diseased liver (Clare, 26). In both cases, the phototoxic response occurs due to the presence of the agent in the dermis or epidermis of the skin. In this area the compound can absorb energy causing the photosensitization.

In 1961, Hashimoto reported the photosensitization of humans and cats due to the ingestion of abalone viscera. Initially, he reported that the causative agent, isolated from the liver, was pheophorbide *a*. Further investigation revealed that the compound was pyropheophorbide *a*. This report was the first proof of the sensitization of humans due to the ingestion of chlorophyll derivatives. Due to the interest of our laboratory in the biodegradation of chlorophyll and the health aspects of these substances, we were intrigued by the possible toxic nature of these substances.

Analysis of foods for the presence of primary photodynamic agents will be of increased importance in the future as new foods, derived from plant proteins or produced from single celled organisms, such as algae, become a reality. In the utilization of these foods the possibility exists for increased levels of porphyrin intake, due to their concentration during the processing of the foods. If the liver of the consumer were taxed beyond its ability to detoxify the porphyrin compounds from the body, upon exposure to the proper intensity and wavelengths of light, photosensitization would be a certainty. Testing for the presence of photodynamic agents should be included in the development of these new foods.

Presently, the concern was to discover what foods, if any, provide concentrated levels of potential photodynamic agents. The liver of the abalone was one food containing such compounds. Foods derived from organs having similar functions might be potentially dangerous. A system was needed for assaying the extracts of such foods for photodynamic agents. Such a system must be sufficiently sensitive for the analysis of small quantities of these agents, and it must be reproducible.

A study of the toxicity of pyropheophorbide *a* was undertaken to develop a bioassay system for the analysis of derivatives of chlorophyll, pyropheophorbide *a* and *b*, pheophorbide *a*, and pheophytin *a*. This bioassay provides a method for the analysis of phototoxic substances in foods and enables us to make dietary recommendations relative to these assays. The analysis for phototoxic substances in livers from domestic animals were included in the objectives of this study.

LITERATURE REVIEW

Photodynamic Action

The essential nature of the sensitivity of organisms to light was first shown in 1900. O. Raab (81), who was studying the toxicity of acridine dye toward paramecia, noted an inconsistency in the time required to kill the organisms at constant dye concentrations. He showed that the time variations were related to the intensity of the light to which the organisms were exposed. The dye rendered the organisms sensitive to light in a manner similar to the sensitization of a photographic plate. Within a relatively short period of time many dyes and pigments were discovered to possess this sensitizing ability. Eosin and chlorophyll were included among the early discoveries (Blum, 15).

Initially, the term photodynamic action was given to sensitization phenomena by Tappeiner and Jodlbauer (99), who thought this phenomenon was the basis of photobiological processes in general. Its actual significance in photobiology has been more limited than originally hoped. Currently, photodynamic action is used exclusively to describe phenomena of the type observed by Raab (15).

H. F. Blum (15) has written one of the most complete monographs on the subject of photodynamic action. He surveyed the use of photodynamic action as a model system for the elucidation of other photobiological processes, the sensitivity of human beings and animals to light, the development of sensitivity to light as the result of the

administration of photodynamic substances as therapeutic or diagnostic agents, and the elementary principles of photochemistry.

Another major review of the properties of photodynamic agents was presented by Santamaria and Prino (88). This review covered the essential properties of eighty known photodynamic substances showing high correlation between photo-efficiency and carcinogenesis. It also discussed the natural photodynamic sensitivity in cells and tissues. They noted the importance of these natural photodynamic systems in producing damage to the retina, sperm, or tumor cells under prolonged exposure to light.

Blum (15) used the term photodynamic action to designate the oxygen dependent, lethal or inhibitory effects exerted by sensitizing agents on living and non-living biological systems which have been irradiated with ultraviolet and visible light. Fowlks (45) proposed the term photosensitization because the oxygen requirement imposed by Blum was untenable, when looking at biological processes in the broadest sense (38). Photosensitization, he proposed, should be the term for describing both the oxygen dependent and oxygen independent effects produced in biological systems in the presence of light and a sensitizing agent.

Fitzpatrick and others (41) indicated that the photosensitized reactions elicited in humans and animals by chemical agents are dependent on the following factors:

a. Absorption of light by the agent and the skin,
b. Structure and photoreactivity of the agent,
c. Wavelength of light used in irradiation,
d. Amount of effective photic energy absorbed and the duration of the exposure,
e. Dose and concentration of the agent,
f. Solubility and penetrating capacity of the photosensitizer and its ability to combine chemically with a sensitive cell constituent,

g. Amount of melanin normally present in the skin, and
h. Erythemal threshold of the individual.

Further, they felt that a unified mechanism of photosensitization at the molecular level will emerge as additions are made to understanding a.) the nature of photo-activated singlet and triplet molecular states of photoexcited compounds, b.) the nature of unpaired electrons (free radicals) generated during illumination, and c.) the nature of mechanisms by which energy is transferred in biological systems.

Smith and Jones (96) relate that, historically speaking, the condition known as photosensitization has been recognized in farm animals for years, especially, but not exclusively, in sheep and cattle. The clinical manifestations include itching sensation, erythema, and inflammatory edema. The inflammation was often so severe that the skin died, and the necrotic layer sloughed off with time. These changes are strictly, and often sharply, limited to areas of the body surface that are 1.) in a position to receive the direct rays of sunlight and 2.) are unprotected, lacking pigmentation, or a thick coat of hair.

According to Radileff (82) photosensitization may be of four types: 1.) a dermatitis, with swelling, due to the consumption of photodynamic substances (primary); 2.) sensitization as a result of, or concomitant with, liver damage (icterogenic); 3.) sensitization as a result of the ingestion of a photodynamic chemical administered as a medicament; or 4.) congenital photosensitivity, which is believed to be hereditary in origin. The sensitization due to photodynamic agents from plants results from the manifestation of lesions and injury in the unpigmented areas of the skin. The ingestion of certain plants leads not only to photosensitization, but also to kidney and liver damage, jaundice, and death in some cases.

Phylloerythrin, a porphyrin structurally similar to pyropheophorbide *a*, is an active sensitizing agent produced from chlorophyll by the ruminant microorganisms (82). Normally, it is removed from the animal through the liver and excreted with the bile. If the liver is deranged, phylloerythrin excretion is reduced, and the compound appears in the skin as a photosensitizer. Rimington and Quin (85) were the first to show that phylloerythrin was the actual agent causing *geeldikkop* or "thick-head" disease in cattle and sheep.

Clinical symptoms of photosensitivity, stated Cripps (31), are produced by a photochemical reaction in the skin. Numerous requirements must be met for the reaction to occur. The photosensitizer must be in the epidermis or dermis and in sufficient concentration at the time of irradiation. The exciting wavelengths must be of sufficient intensity at the time of irradiation. These wavelengths must also be in the range that the photosensitizer will absorb. Baer (8) has discussed the processes involved in the production of photosensitivity.

Magnus (70) claimed that one of the most important matters requiring investigation in photodermatoses is the determination of the action spectrum (i.e., the identification of the wavelengths of light that provoke changes in the skin). This information is useful for two reasons: First, it enables the clinician to choose the proper therapeutic for application to the skin to prevent penetration of the wavelengths which specifically initiate the symptoms. Second, the knowledge of the action spectrum of a disease might lead to an understanding of its pathogenesis or even to the identification of the photosensitizing agent. Hashimoto and Tsutsumi (50) used the action spectrum for the sensitization induced by abalone livers as an indication that porphyrins

might be involved. This knowledge led them to the isolation of pyropheophorbide *a* using a classic porphyrin extraction procedure.

Magnus, Porter, and Rimington (69) demonstrated that the action spectrum for the edematous component in the photosensitization produced by porphyrins coincided with the positions of their Soret bands and leaves little doubt that it is an excited form of the photosensitizer which is effective in this respect.

As recently as 1963, the exact site of action *in vivo* was not known (41). Fitzpatrick and others (41) reasoned that the photic energy possibly acts on the cell membrane, cytoplasm, cytoplasmic organelles (mitochondria, microsomes, and lysosomes), or nuclear tissue. Since that time, Allison and others (1 and 2) and Slater (94) have shown by fluorescence microscopy and cell culture techniques that substances such as anthracene and porphyrins are concentrated in lysosomes. If these substances absorb light, the lysosomal enzymes escape into the cytoplasm and kill the cell with their lytic action.

While photosensitization can occur as the result of therapeutic treatments, most cases are aggravated by sunlight. Sunlight, as it reaches the earth, presents a continuous spectrum of electromagnetic radiation. The ozone barrier (15-35 km above sealevel [87]) filters out all of the ultraviolet radiation between 200-290 nm and most of the radiation of the sunburn spectrum, 290-320 nm. This range of the spectrum is responsible for 99% of the sunburn response (37 and 17). The visible sensation of light lies within the 360-650 nm range. The range greater than 650 nm constitutes the infrared area of electromagnetic radiation. In the case of porphyrin induced photosensitization, the principal wavelengths of light responsible for the lesions are the

region of 400 nm (69). The distinction can be made between sunburn and porphyrin sensitization by testing the person or animal under radiation which has passed through window glass. If sensitization occurs from this exposure, the visible range of the photic energy is implicated as the causative energy.

Blum (15) enumerated three requirements to be met in the investigation of a suspected photosensitivity disease. These are: 1.) The symptoms of light sensitivity must be elicited by exposure of the animals to sunlight, preferably to sunlight through window glass. 2.) A photodynamic substance must be isolated in a pure form which will produce the symptoms if injected into the experimental animals only when followed by exposure to light. 3.) It must be demonstrated that the wavelengths which produce the sensitivity in postulates 1 and 2 are identical.

Clare (25) enumerated several practical considerations in discussing the application of these requirements to actual experimentation. He and Blum emphasize that any substance isolated from material ingested by animals must be shown to be effective by oral administration. The value of action spectra, he continues, is seriously limited by difficulties in determining them with sufficient accuracy. When the very wide range of the absorption spectra of pigments such as porphyrins and their close similarity are considered, the difficulty of even reasonably precise equation of the action spectrum with the absorption spectrum of a possible photodynamic agent is increased.

Photosensitization Induced by Pyropheophorbide *a*

Photosensitizations in cattle and sheep resulting from abnormalities of porphyrin metabolism or with liver dysfunctions which permit

accumulation of sensitizing chlorophyll derivatives has been known for years (Rimington and Quin, 85; Blum, 15; Clare, 25, 26, and 24). Humans have also been shown to be sensitized by abnormal amounts of porphyrins (Meyer-Betz injected himself with 200 mg of hematoporphyrin and was sensitized when exposed to sunlight [Clare, 26]). Clare (24) classified photosensitization on the basis of the origin of the agent or the process by which it reaches the skin. In animals these photosensitivity diseases are of two types. In Primary Photosensitivity the photodynamic agent is a plant pigment not usually encountered in the diet of the animals and is not efficiently excreted or detoxified by the liver; in Hepatogenous Photosensitivity the agent is the chlorophyll breakdown product phylloerythrin. Normally, this is absorbed from the digestive tract and excreted in the bile. Only when the excretory function of the liver is impaired does phylloerythrin accumulate in the blood and reach the skin.

Letham and Clare (as cited in 24) were the first to demonstrate that chlorophyll derivatives or other pigments with a porphyrin structure, administered orally, can produce photosensitization without a prior disturbance of liver function. Further, they were the first to implicate pyropheophorbide *a* as a photodynamic agent. It was not, however, a natural component of the green millet they were feeding, but it was formed as an artifact of the drying and extraction processes they were using (24).

Hashimoto and others (50) reported that, before a prohibition period was legislated against abalone fishing, the inhabitants, as well as the cats, of the Japanese coastal cities suffered from a dermatitis if abalone was eaten during the months from April to October.

Hashimoto screened abalone liver in a bioassay system and reported the toxic characteristics of this tissue (50). Using classic procedures for extraction of porphyrins, Hashimoto extracted the liver material and assayed the fractions for phototoxic properties. The material isolated in one fraction showed phototoxic properties and Hashimoto and Tsutsumi reported the compound initially as pheophorbide *a* (51). In 1964, Tsutsumi and Hashimoto (112) reported that after further investigation the sole photodynamic agent in the liver of abalone was pyropheophorbide *a* and retracted their earlier claim.

Chlorophylls

Chlorophyll chemistry has been reviewed many times. Two complete and recent reviews are by Arnoff (5) and Vernon and Seely (115). Arnoff related the chemistry of chlorophylls to those changes observed in processed foods. Vernon and Seely have edited a work which covers chemical structure, extraction, spectral properties, analytical procedures, and function of the pigments in photosynthesis.

Systems for the nomenclature of the chlorophyll molecule are given in Arnoff, Vernon and Seely, and Holt and Jacobs (54). An adaptation of the Holt and Jacobs system for representation of the derivatives is presented in the Methods section.

The procedures for preparation of the derivatives of chlorophyll were taken from Strain and others (107, 108, 109, 110), Holden (52 and 53), Seely (91), Anderson and Calvin (4), Pennington and Strain (79), Vernon (114), Willstätter (120), Stoll and Wiedemann (106), and Fischer (40).

Data on the visible spectra of chlorophyll *a* and *b* were obtained from Strain (107) and Smith and Benitz (97); pheophytin *a* and *b*

from Rimington and Quin (85), Zscheile and Comar (125), and Stanier and Smith (101); pheophorbide *a* and *b* from Tsutsumi and Hashimoto (112), Todd and Galston (111), and Stern and Wenderlein (102-105); pyropheophorbide *a* and *b* from Tsutsumi and Hashimoto (51), Todd and Galston (111), Stern and Wenderlein (104), and Fischer (40); and phylloerythrin from Stern and Wenderlein (103) and Rimington and Quin (85). Holt and Jacobs (54), Weigl and Livingston (117), Pennington and Strain (79), and Hashimoto (personal communication to Dr. S. H. Schanderl) provided the information for the interpretation of the infrared data.

Photochemistry

Photosensitization results from the interaction of light, sensitizer, and biological organism. Blum (15) showed that the photochemical processes are oxygen dependent and temperature independent.

Lamb (63) has delineated the processes through which photobiological reactions occur. His parameters are:

1. Light must be absorbed to produce a photochemical reaction.
2. To be absorbed, the energy of the photon must be equivalent to the energy of some energy transformation of the absorbing molecule.
3. One quantum of light will be absorbed by and activate one molecule in a primary process.
4. In complicated molecules, energy absorbed in one portion of the molecule may be transferred to other atom pairs and cause the reaction to take place in a different part of the molecule than the absorbing portion.

Fowlks discussed the physical-photochemical principles involved in photosensitization. He stated (45) that a beam of light which traverses a body of matter unchanged either in intensity or wavelength cannot cause any chemical or physical change in that body of matter. This is in agreement with the law of photoequivalence and the Grothus-

Draper Law. Conversely, he continues, if a beam of light traverses a body of matter, but, after allowing for scattering, is found to be reduced in intensity, the wavelengths of light which do not emerge have made some physical or chemical changes or both in the body of matter. Photodynamic action is a chemical change, and those absorbed wavelengths which cause chemical change will include the photodynamically effective wavelengths.

Kirshbaum (61) has stated that photosensitizers are chemical compounds which, on absorption of energy, induce a photochemical reaction. This photochemical event is a primary event and obeys the Bunsen-Roscoe reciprocity law (45).

The principle absorbers are molecules which possess the pi-electron system (41). Energy absorption activates these molecules raising the energy level of some valency electrons to short lived (10^{-8} sec) excited states. What happens after activation depends not only on the nature of the absorbing molecule and the amount of energy in the absorbed quantum, but also upon environmental factors and the probability of collision with neighboring molecules. These excited molecules have many routes for further reaction. They may:

a. Lose energy as heat,
b. Dissipate the excitation energy as fluorescence,
c. Transfer the energy to other molecules,
d. Dissipate excitation energy in the form of phosphorescence (indicating that the activated state is a reactive metastable in which a pair of electron spins is uncoupled to have parallel spin),
e. Induce the formation of free radicals, or
f. Cause ionization, dissociation, and other physical interactions (41).

Biological tissue may respond to light resulting in denaturation, sensitization, mutation, and death.

Pathak (78) and Fowlks (45) discuss the manner in which photochemical reactions manifest their changes in organized biological systems and deal with processes similar to those outlined above.

Bellin (12), discussing the photochemical properties of bound photosensitizers, has stated that mechanisms have been postulated. She indicates that it is the increased probability of transition to the metastable state by increased spin-orbital coupling, and energy transfer between the absorbed dye molecules in the ground state and those in the first electronically excited singlet state that account for the reactivity of the sensitizer. Enhancement of the metastable (triplet?) electronically excited state bears an important relation to the altered behavior of dye molecules in photoreduction. This phenomenon, she continues, may also be of importance in explaining the observed transfer of electronic energy, which can occur in many biological systems. It is the metastable state of the chromophore which is most probably involved in the ultimate energy transfer from the pigment to a substrate molecule. She proposed the following scheme as the probable reaction sequence experienced by a photosensitizer:

1. $D + h\nu \longrightarrow D^*$
2. $D^* \longrightarrow D' + \text{Heat or } h\nu_f$
3. $D^* \longrightarrow D'$
4. $D^* + D \longrightarrow D' + D$
5. $D' \longrightarrow D + h\nu_p$
6. $D' + A \longrightarrow D + A$
7. $D' + A \longrightarrow M$
8. $M \longrightarrow \text{Colorless}$

D^* = First electronically excited state
D' = Long lived electronically excited state
$h\nu_f$ = Fluorescence
$h\nu_p$ = Phosphorescence
$h\nu$ = Light
D = Dye or pigment
A = Quencher of long-lived excited state
M = Product

Foote (44), Spikes and Glad (98), Spikes and Straight (100), Spikes (99), Blum (15), Simon and Vunakis (93), and Fowlks (45) all proposed reaction sequences, similar to the one above, to explain the chemical processes involved when activated molecules exhibit their photodynamic action.

Work on the macromolecular level of mechanistic investigations has shown that where proteins are damaged peptide bonds are not ruptured by photodynamic treatment, but the damage results from the destruction of amino acid side chains (100). Information on the sensitized photoautoxidation of free amino acids indicates that histidine, tryptophan, tyrosine, methionine, and cysteine residues are the principal loci of photodynamic damage to proteins (100). The changes in proteins due to this damage are observed in conformation, electrophoretic patterns, viscosity, solubility, surface tension, and sedimentation behavior. Further studies on photodynamic damage to free amino acids was reported by Weil (118), Fowlks (45), Simon and Vunakis (93), and Sluyternian (95). Inactivation of coliphage, mechanistic proposals, and kinetic studies involving protein damage are given by Yamamoto (123 and 124).

Ke and others (60) have studied the excited states and energy transfer capability of chlorophyll. Oster (77) noted that only those compounds capable of being photoreduced can act as sensitizers for photooxidation. Both reactions proceed through metastable, long-lived excited states. The sensitizer in these reactions is not consumed in the overall process and may be used over and over again as long as some autoxidizable substrate remains. Mauzerall (73) stated that chlorophyll and its derivatives which have an active "methylene" group at position 10 possess the capability to act both as a photooxidant and photoreductant.

All of the mechanisms presented above involve close range molecular interactions. Fitzpatrick and others (41) urged that mechanistic studies should not overlook the possibility that "conduction-band" and long-range dipole-dipole transfer can also be responsible for the transport of photic energy from the absorption site to a somewhat remote region (10 Å) before it is used or dissipated. Energy can be transferred by molecules, such as proteins, which are periodic (pseudo-crystalline) in nature. Photoconduction has been shown to take place in crystalline (periodic) materials (41). A hydrogen bond network can also act as a conduction band. Long range dipole-dipole transfer provides a means for transferral of absorbed energy over considerable distances between identical and non-identical molecules.

Retinal Lesions

Crews (30) stated that the retina is well suited for study *in vivo* or as an isolated organ. The eye often acts as a sentinel, being the first part of the body to show toxic effects in many instances. Deleterious effects are more severe if they involve the posterior segment of the eye or the visual pathway.

Cases of damage to the eye caused by photosensitization, particularly the retina, are not numerous. Jaffe (55) reported that retinal hemorrhage and bilateral oculomotor nerve palsy were observed in a case of acute porphyria, a hereditary disease resulting from increased levels of heme porphyrins in the blood. Barnes and Boshoff (9) reported fifty percent instance of ocular lesions in porphyria patients. Wolkowicz (122) reported ophthalmoscopically observable retinal lesions in rabbits sensitized with eosin. These lesions were described as resulting from the accumulation of fluid (exudate below the retina

producing detachment). The most serious lesions were confined to the choroidal layer and pigment epithelium, and the retina secondarily. A time study of their development showed a slow absorption period producing swelling and separation of nerve fibers, formation of large globular cystic spaces in the outer plexiform layer, ultimate swelling and disintegration of the rods and cones, and hole formation. Wolkowicz cites the work of Hei-Uko, who produced similar lesions with eosin and tripaflavin.

Noell and others (76) reported damage to the rat retina induced by monochromatic light, green (1200-2500 lux), after exposures of not greater than 24 hours. They reported the lesions as irreversible reduction of the electroretinogram amplitudes and degeneration of the visual cells and pigment epithelium. They also postulated a causative mechanism.

Gerstein and Dantzker (46) reported retinal vascular changes in rats suffering from hereditary visual cell damage. Dantzker and Gerstein (32) subsequently reported retinal vascular changes following toxic effects on visual cells and pigment epithelium. They required high intensity light and iodoacetate to destroy the cells.

Miscellaneous references have been collected regarding the damaging effect of light upon the eye. Duke-Elder (35) reported that the pathological effects of light on the eye were caused by overstimulation, thermal action, and abiotic action. His compendium (36) discusses nearly all facets of retinal diseases. Bachem (7) discusses the ophthalmic effects of ultraviolet light. The action spectra for corneal keratitis revealed that 282 nm was the wavelength producing the greatest effect. He discovered that the spectral range for

production of cataract is 254-310 nm. Clark (27) dealt with damage to the eye caused by accidental or occupational hazards and the light transmission properties of the tissues of the eye. Cloud and others (28 and 29) reported the incidence of corneal lesions produced by photosensitization with methoxsalen, a furocoumarin. They also describe the gross appearance of the eye as a result of the sensitization.

Occurrence of Photodynamic Agents in Foods

At this time, the study of abalone by Hashimoto and others (50) and Tsutsumi and Hashimoto (112) is the lone report of the ingestion of a food containing a phototoxic agent which subsequently caused primary photosensitization of the individual. Jones and others (57) report that chlorophyllides and pheophorbides are formed in appreciable quantities in okra, turnip greens, and other green vegetables as a result of blanching at 180° F during processing.

Phylloerythrin, formed in the rumen from chlorophyll, has structural similarities to pyropheophorbide a. It has been reported as being present in the blood of sensitized animals (80), dispersed on the colloidal micelles, as are the bile salts and plasma proteins. In support of this claim is the observation (Rimington and Quin, 85) that phylloerythrin was present entirely in the plasma of sheep affected with *geeldikkop*. Only traces were found in the well-washed corpuscles.

Terms

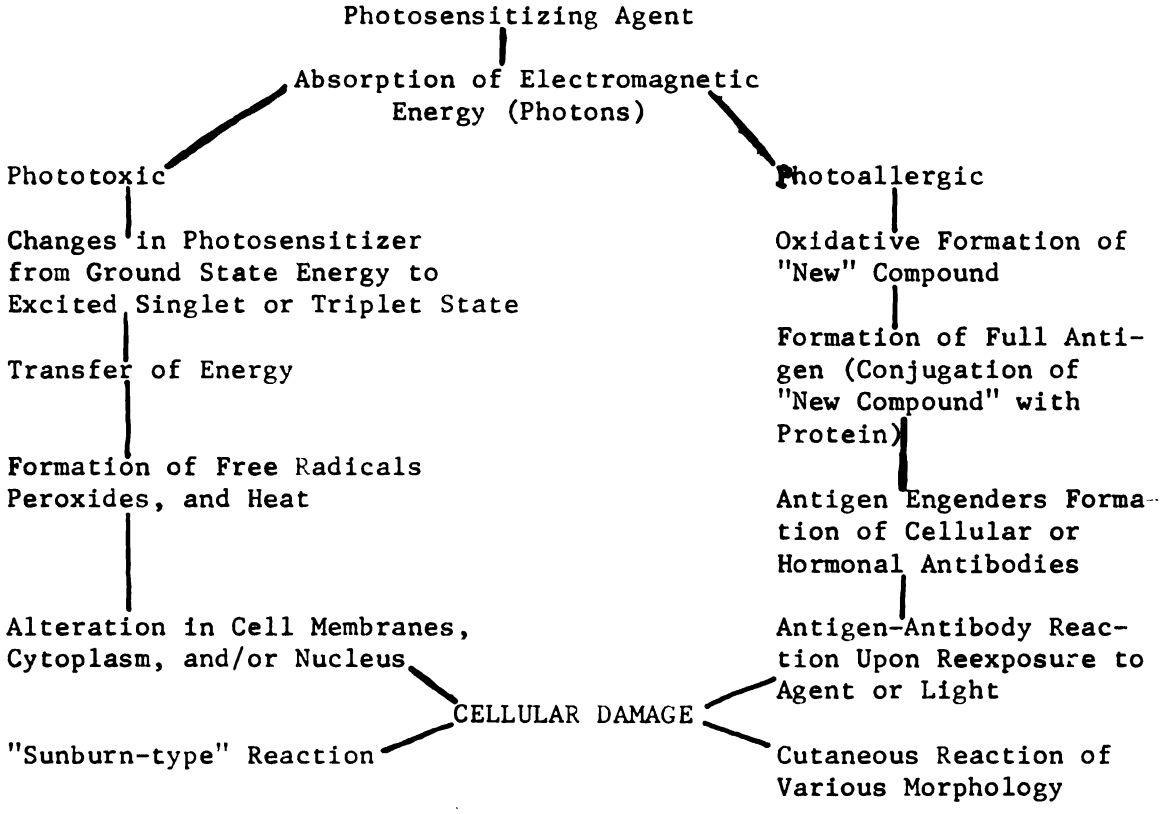
Technical terms describing photodynamic action and diseases caused by light have been the focus of considerable controversy (Blum, 15; Epstein, 38; and Fowlks, 45). For the purpose of this study, I

will define the terms pertinent to a clear discussion of the problem and results. Epstein (38) has given the most comprehensive explanation of photosensitization, stating that this process refers to activation of a molecule by light, and the transfer of energy to another molecule, causing it to dissociate or to react chemically. This process is not necessarily restricted to the description of changes in a system of biological material and sensitizer. Photobiological reactions are photochemical reactions in biological materials.

Photodynamic action was described by Blum (15) as being limited to photosensitizations requiring oxygen. Epstein (38) expanded the scope of the meaning to include more than the single process of energy absorption. In its widest usage, it describes those photobiological reactions which are based on the transfer of energy from one light absorbing activated molecule to another. It is more correct to apply the term to all photobiological processes. Epstein stressed that allergy and toxicity refer to photosensitivity diseases. Phototoxicity is elicited in all individuals when the right amount of sensitizer is used, and the proper wavelength of light is present. The reactions vary in direct proportion to the dosages. Photoallergy is the disease which results from the light induced changes of a normal metabolite or a foreign substance which acts as a true allergen sensitizer. Lamb (63) indicated that photoallergies occur in only a few individuals. They react with the formation of urticarial or papular lesions upon testing the skin with light. There is no specific wavelength required for the inducement of the response. Usually, there is an incubation period and a delayed response which may be papular, eczematous, or urticarial. Baer (8) has provided a schematic analysis of the

differences between phototoxic and photoallergic responses. This scheme is found in Figure 1.

Figure 1. Schematic analysis of differences between phototoxic and photoallergic responses.



MATERIALS

Chlorophyll

Frozen California spinach was obtained from the Michigan State University Food Stores, dried in a Proctor and Schwartz cabinet drier, and extracted to yield the chlorophyll which served as raw material for the synthesis of other pigments used in this study.

Light Source

A twelve tube bank of fluorescent lights (Sherer-Gillett Company of Marshall, Michigan) was used as the light source. The tubes were Sylvania Cool White Power-tubes F48T 12-CW-VHO. This bank was arranged as shown in Figure 2. The center of the bank was positioned nineteen inches above the platform. The spectrum for this light source at this distance is given in Figure 3. The intensity was 18,000 lux for the small platform, and 21,000 lux when a larger platform was used.

Exposure Cages

In the early studies, the animals were placed in inverted galvanized animal cages with wire mesh on two sides. Later, to decrease the shade areas, inverted, polystyrene mouse cages were employed, fitting five to a board, as shown in Figure 2.

Chemicals and Solvents

The chemicals used as vehicle in the injections were propylene glycol (Eastman Organic Chemicals #1321) and DMSO (K and K Laboratories

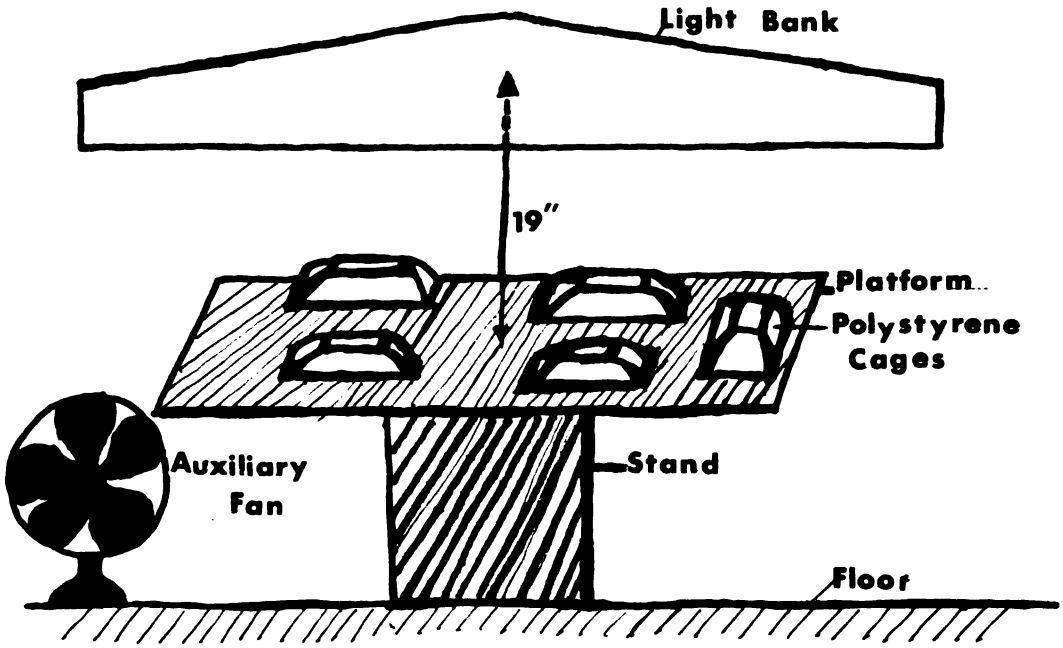


Figure 2. Exposure apparatus and arrangement.

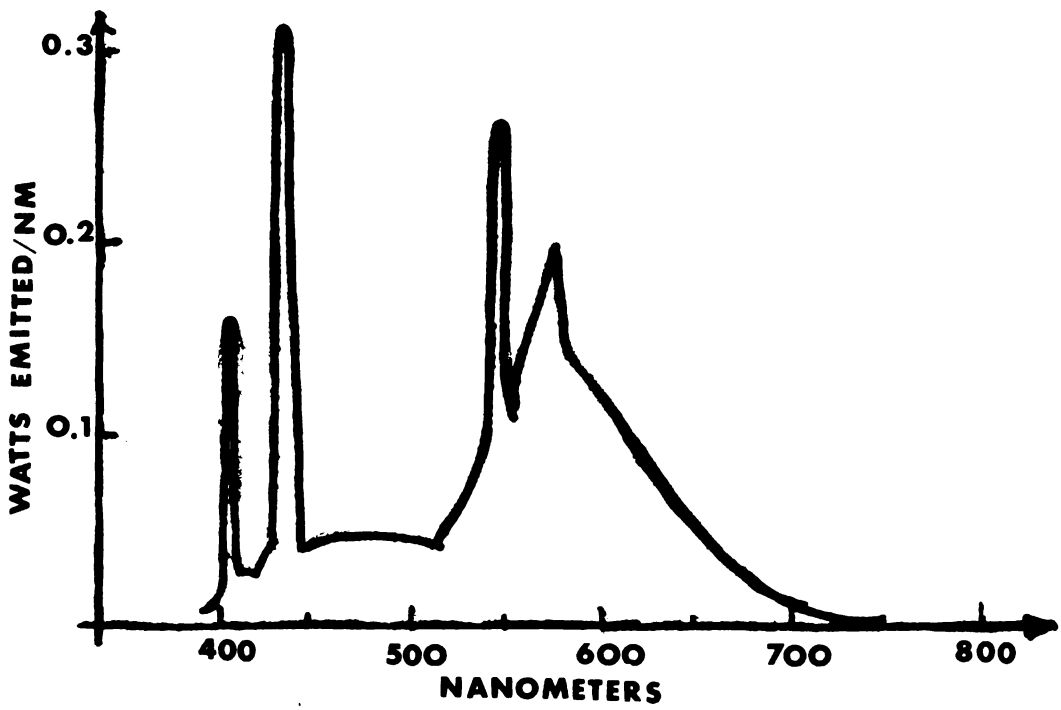


Figure 3. Distribution curve of spectral energy for the light bank.

#5147). All solvents used were ACS Reagent Grade and were used without further purification, unless noted in the procedure.

Rats

The rats used in this study were from Sprague-Dawley stock, obtained from Spartan Research Animal, Incorporated, Haslett, Michigan.

Histological Preparations

The histological preparations were made in the laboratories of the Department of Pathology at Michigan State University.

Grinding

The dried spinach was ground on a Fitzmill, Model D, Comminuting Machine, The W. T. Fitzpatrick Company, Chicago, Illinois.

Drier

A Proctor and Schwartz Pilot Plant sized cabinet drier was used to dehydrate the spinach.

Spectra

Visible spectra were measured on a Beckman Model DK-2 Recording Spectrophotometer. The IR measurements were made on a Beckman IR-12, double beam infrared spectrophotometer.

Light Intensity and Spectral Distribution

A Weston Illumination Meter was employed to measure the intensity of the light source. The 756 Model is a product of the Weston Instrument Division of Dagstrom, Incorporated, Newark, New Jersey.

An ISCO Model SR Spectroradiometer, which measures spectral distribution and intensity of lightwaves in micro-watts per sq cm per

nanometer, permitted the measurement of intensities from 380 to 1050 nanometers. The radiometer is produced by the ISCO Instrumentation Specialties Company, Incorporated, Lincoln, Nebraska 68507.

Livers

Beef and pork livers were obtained from the Michigan State University Meats Laboratory to analyze for phototoxic agents. The animals from which the livers came had been raised by Michigan State University livestock researchers on diets comparable to those of commercially raised animals.

METHODS

Oral Feeding of Pyropheophorbide

Bread wafers covered with a solution of pyropheophorbide in palm oil provided the best means to administer the compound orally. The wafers were prepared as follows: The dose of compound to be given was weighed out and placed onto a metal, one-fourth teaspoon measuring spoon. The material was dissolved in acetone, and the acetone driven off by heating the spoon on an electric hot plate, producing a film of pyropheophorbide on the bowl. Palm oil, which had been heated, was poured into the bowl to dissolve the pyropheophorbide. Once dissolved (solution was indicated by fluorescence of the compound under long wavelength ultraviolet light), the material on the spoon was solidified by placing it in the refrigerator. When the mass had solidified, it was spread on one or two small circles of bread cut from a slice of bread with a number 12 cork borer. The material remaining on the spoon was scoured off with powdered sugar, and the sugar transferred to the bread. Scouring the spoon in this manner aided in quantitative transfer of the dose. These pyropheophorbide sandwiches were stored in the refrigerator to prevent loss of the compound by weepage.

The sandwiches for each oral feeding were given to rats which had been starved for 24 hours. This procedure assured rapid and complete ingestion of the material with only the slightest loss of material on

the cage floor, rat paws, or face. Doses as large as fifteen milligrams have been successfully fed by this method.

Other attempts were made at oral administration using a pH 10.3 buffer, vegetable oil and ethyl alcohol (after Hashimoto and others, 50), and bread wafers to which the pigment was applied with solvent and the solvent was then evaporated. All of these oral methods were considered inefficient or ineffective for our purposes.

The pigment was also administered orally by dissolving it in a high fat diet provided by Dr. O. Mickelsen of the Michigan State University Department of Foods and Nutrition. Pigment given in this manner did not improve the efficiency (reduction in the amount of the pigment to produce a toxic response) of our oral feeding.

Stomach Tubing

Experiments were conducted in an attempt to develop a means of quantitatively dosing the animals by stomach tube. Suspensions of pyropheophorbide, ethyl alcohol and vegetable oil were used to gavage the animals. These animals showed only minor signs of sensitization; therefore, stomach tubing was discarded as a means of dosing.

Liquid

Hashimoto reported that the pyropheophorbide was soluble in pH 10.3 phosphate buffer. Attempts were made to solubilize the compound in this buffer. Since fluorescence of the material did not appear when the buffer was used as solvent, this vehicle was not used as a means for oral administration.

Injection Administration of Pyropheophorbide

Intraperitoneal and intravenous injections were tried in an attempt to reduce the quantity of pigment required in the assay. Accomplishing this goal facilitated the administration of materials. Various vehicles were used in establishing the parameters for these procedures.

Ratios of carboxy-methyl cellulose, dimethyl sulfoxide (DMSO), and/or propylene glycol (1,2-propanediol) were used. None of these three could produce photosensitization through intraperitoneal injections. Postmortem examinations of animals injected in this manner revealed that the material precipitated out in the peritoneal cavity and was not assimilated by the animal. For this reason, assays by peritoneal injections were discontinued.

Intravenous injections of the compound in a vehicle containing 0.4 milliliters (ml) of propylene glycol and 0.02 ml of DMSO provided the most successful means for pigment assay. Studies revealed that sensitization could be obtained with 1 mg of pyropheophorbide a. The upper limits for this assay were doses of 4 mg. Greater quantities of pyropheophorbide precipitated in the heart and lungs causing respiratory shock and death.

A summary of the results of the preliminary studies appears in Table 1.

Exposure of the Animals

To perform the bioassay, an exposure system was developed utilizing a light source producing 20,000 lux from 12 cool white fluorescent tubes. Polystyrene mouse cages were inverted on a plywood board to confine the rats, which were given complete freedom of motion. The

platform holding the rats was positioned in the center of the light bank and nineteen inches below the reflecting surface of the bank. The fan in the light bank and an auxiliary fan were used to reduce the temperature at the board surface eliminating the chance of death caused by heat.

Bioassay System

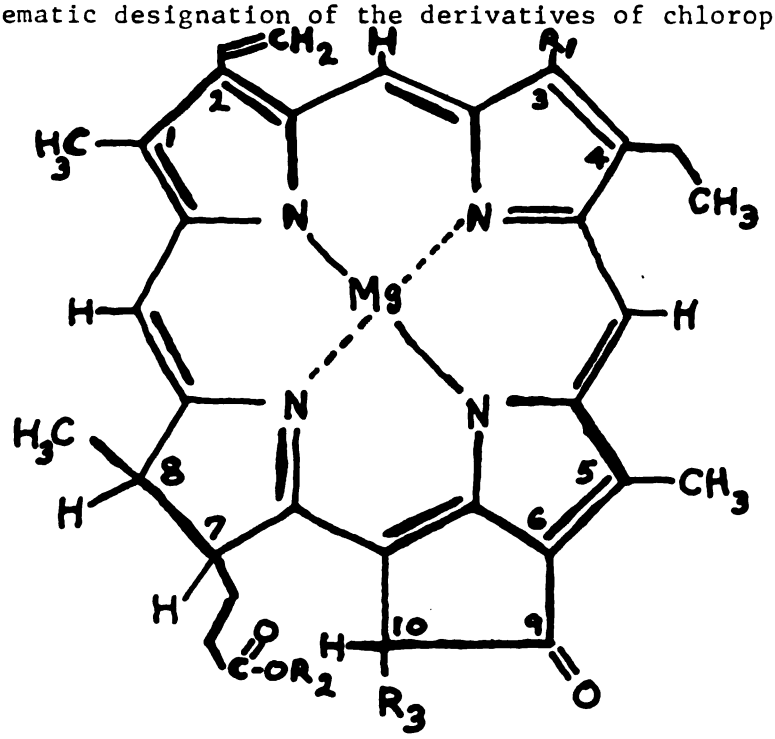
The bioassay system which was developed involved the following procedures: The pigment dose was dissolved in 0.4 ml propylene glycol, 0.02 ml DMSO, and injected into the lateral tail vein of the rat. The animals were held in the dark for 24 hours before exposure to light and given food and water *ad libitum*. To expose the animals, they were placed under inverted polystyrene cages, which were fastened to a plywood board. Water was provided. The platform, holding as many as five animals, was placed on a stand centered under and nineteen inches below the lights. The lights were turned on for the prescribed period, and the animals were observed for signs of photosensitization.

The length of exposure varied with the design of the experiment. It was dependent on the dosage and the severity of lesions desired. For establishing the parameters of the biological changes for the assay system, the doses were set at 3 mg of pyropheophorbide *a*, and the exposure was limited to two periods totalling three hours.

Preparation of the Derivatives

Holt and Jacobs have provided an efficient method for structurally representing the derivatives of chlorophyll (54). Their system will facilitate a discussion of the chlorophyll chemistry. The system has been adapted to meet the demands of this discussion.

Figure 4. Schematic designation of the derivatives of chlorophyll.



COMPOUND	Mg	R_1	R_2	R_3
CHLOROPHYLL *a*	+	$-CH_3$	Phytyl	$-C-OCH_3$
CHLOROPHYLL *b*	+	-CH=O	Phytyl	"
CHLOROPHYLLIDE *a*	+	$-CH_3$	H	"
CHLOROPHYLLIDE *b*	+	-CH=O	H	"
PHEOPHYTIN *a*	-	$-CH_3$	Phytyl	"
PHEOPHYTIN *b*	-	-CH=O	Phytyl	"
PHEOPHORBIDE *a*	-	$-CH_3$	H	"
PHEOPHORBIDE *b*	-	-CH=O	H	"
PYROPHEOPHORBIDE *a*	-	$-CH_3$	H	H
PYROPHEOPHORBIDE *b*		-CH=O	H	H

Pyropheophorbide *a*

Frozen spinach was dried in a cabinet drier and ground in a Fitzmill. Five hundred grams of the powder was extracted with 100% acetone, the extract was concentrated to 200 ml, and the acetone

concentration was reduced to 70%. The pigments were separated on a polyethylene column, and the pheophytin and chlorophyll bands were dug out and eluted from the solid support with 80% acetone (Holden, 52). The acetone concentration was adjusted to approximately 70%, and the eluate (Fr. I) was ground with *Ailanthus altissima* leaves to provide the chlorophyllase required for the conversion of the pheophytin and chlorophyll mixture to pheophorbides and chlorophyllides.

After three or four hours, the extract was tested by measuring the solubility of chlorophyllide in 0.01 N NaOH. If all of the chlorophyllide was soluble in the NaOH, dephytylation had been accomplished.

The pheophorbide-chlorophyllide (Fr. II) mixture was filtered to remove the remaining leaf tissue. A few grams of oxalic acid were mixed with the porphyrin solution to remove the magnesium, chelated in the center of the chlorophyllide porphyrin ring. A gray solution indicates successful metal removal. The resulting solution of pheophorbides (Fr. III) was filtered and transferred to ether from the acid acetone solution (Fr. IIIa).

Twelve percent HCl was used to extract Fraction IIIa, containing the pheophorbides. This extraction removes unwanted porphyrin derivatives of lower molecular weight. The remaining ether layer (Fr. IIIb) was extracted with 17% HCl until pigment was no longer extracted from the ether. The absence of color in the acid layer was an indication of completion of the extraction. Next, the acid extract (Fr. IIId) was washed with ether, and these colored ether layers were returned to the ether layer (Fr. IIIc), representing porphyrins not soluble in the 17% acid. Once the wash solvent was clear, the acid was overlayered with ether, and diluted to 8% to drive the pheophorbide *a* into the ether.

This ether solution (Fr. IIIe) of pheophorbide *a* was evaporated to complete dryness *in vacuo*.

The pheophorbide *a* was converted to pyropheophorbide *a* by dissolving it in 100 ml of pyridine, the procedure of Fischer (as cited in Holt and Jacobs, 54), and refluxing it for five hours, pyrolyzing the carbomethoxy group on the C_{10} carbon from the cyclopentanone ring.

When the pyrolysis was completed, the cooled pyridine solution was overlayered with ether to extract the pyropheophorbide and washed repeatedly with 100 ml portions of water to remove the pyridine. The ether solution (Fr. IV), when pyridine free, was washed three times with 12% HCl to remove phylloerythrin. Next, the ether fraction was extracted with 17% HCl, transferred back to ether (Fr. IVa), dried *in vacuo*, dissolved in chloroform, and absorbed on a sucrose column. The column was developed with 0.5% propanol, 30% chloroform, and petroleum ether. The pigment band was removed, eluted, repurified by sucrose column chromatography, dried, dissolved in ether, and diluted with petroleum ether until crystals began to form. The pyropheophorbide *a* was allowed to crystallize overnight in the refrigerator and harvested by centrifugation. The crystals were dried *in vacuo* and held at room temperature.

Crystals prepared in the above manner had the characteristics of pyropheophorbide *a*. They exhibited a positive Molisch phase test, and had an absorption spectrum in the visible region identical with the published spectrum (Tsutsumi and Hashimoto, 112; and Holt and Jacobs, 54). The melting point of the crystals was 267° C. The infrared spectrum was measured on the solid pyropheophorbide *a* in a KBr pellet. The absorption bands obtained by this procedure matched bands reported by

Hashimoto (personal communication to Dr. S. H. Schanderl) and Holt and Jacobs (54). The distinguishing feature was that the spectrum of pyropheophorbide *a* lacked the absorption band for the C_{10} carbomethoxy group at 1760-1740 wavenumbers (cm^{-1}). The carbonyl absorption band of the propionate carboxyl was broadened and shifted to 1725 cm^{-1}. The cyclopentanone C_9 ketone band was located at 1695 cm^{-1}, and the 1610 cm^{-1} band was assigned to the C_2 vinyl group. From these data the compound was affirmed to be pyropheophorbide *a*. A schematic representation of the preparation procedures is given in Figure 5.

Figure 5. Schematic representation of the methods employed to produce pyropheophorbide *a* and *b*.

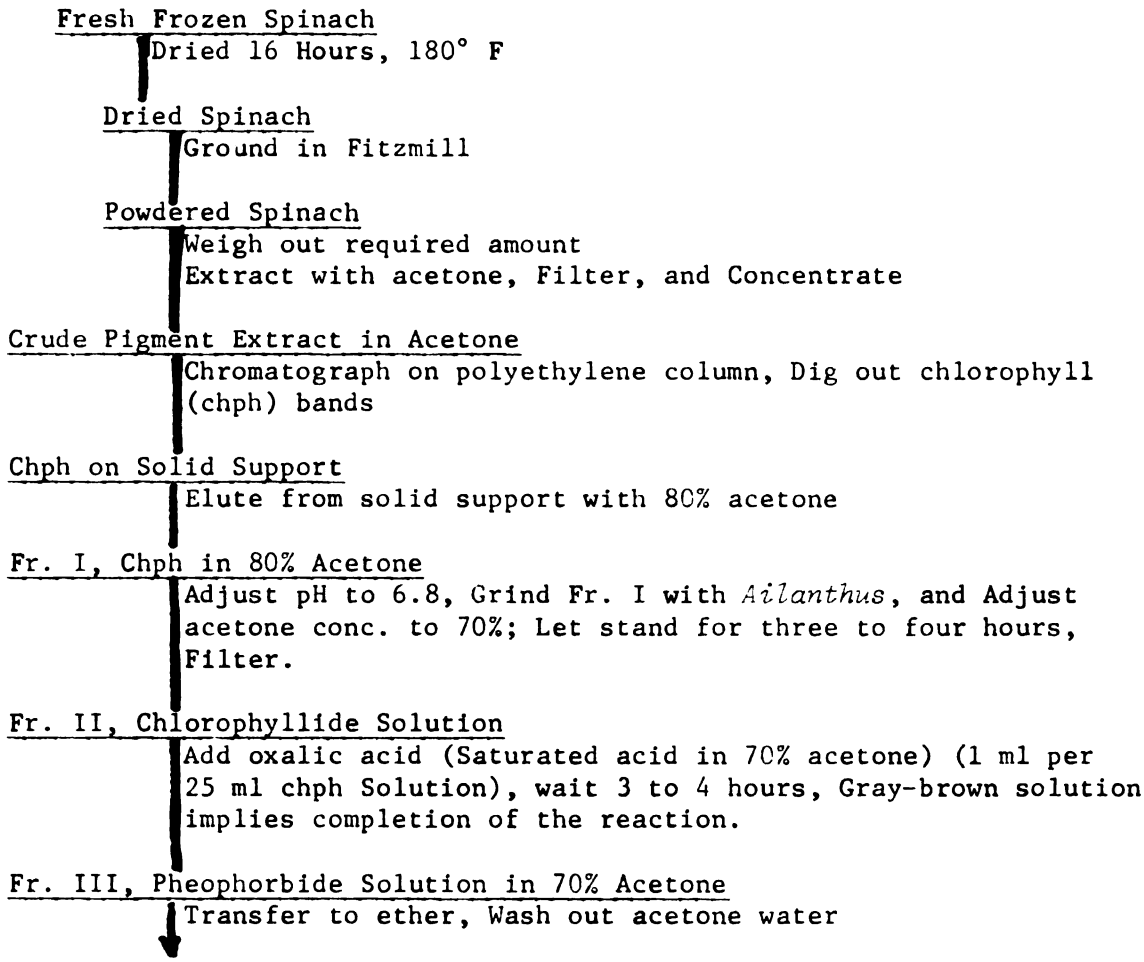
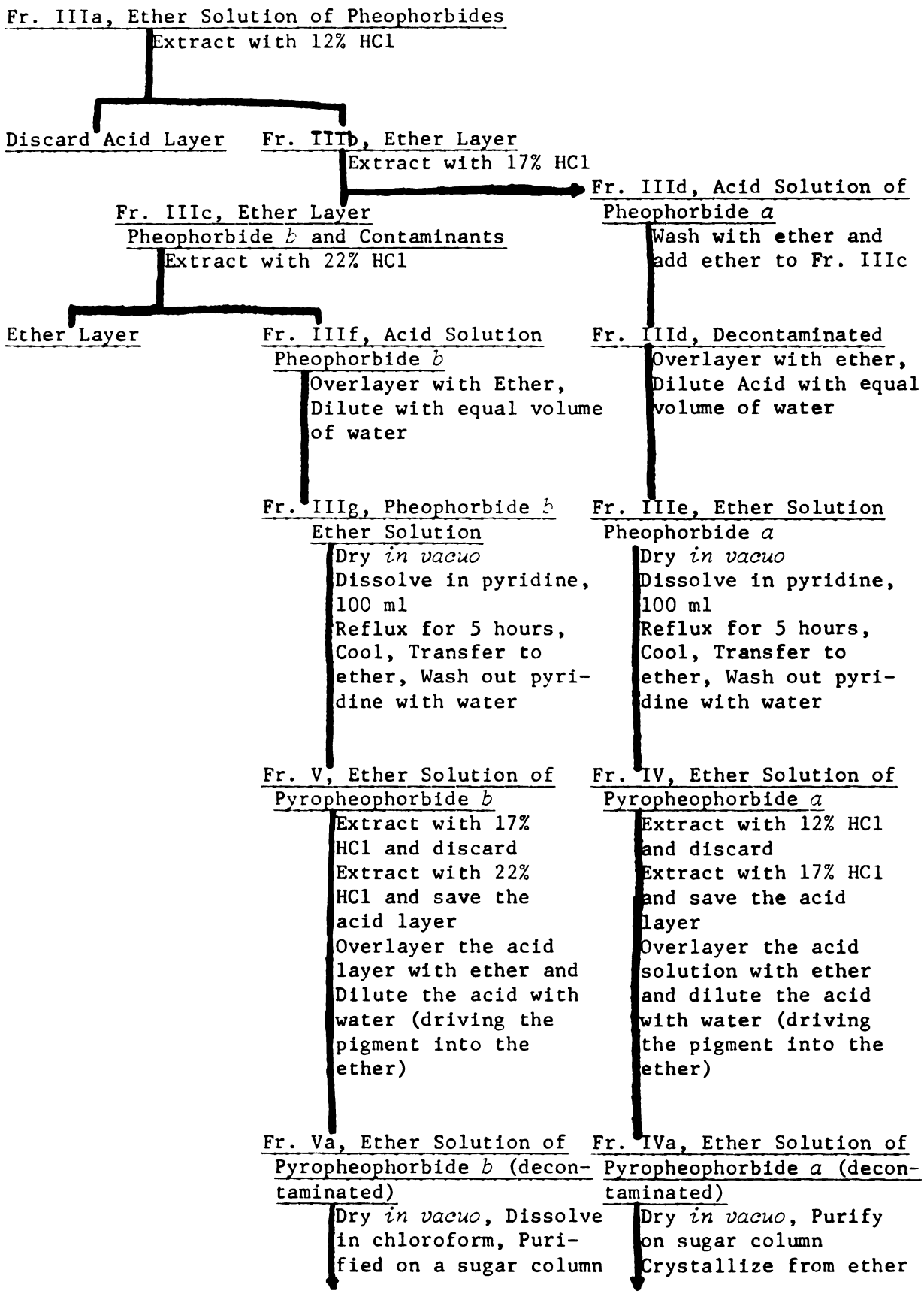


Figure 5 (cont'd)



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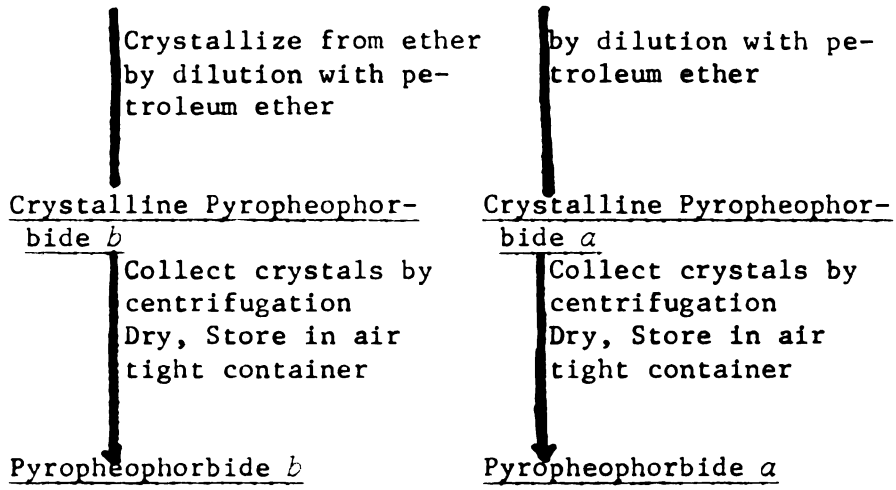


Pyropheophorbide *b*

Pyropheophorbide *b* was prepared in the same manner as pyropheophorbide *a* until the partitioning step (Fr. IIIb). Pyropheophorbide *b* has a HCl number of 19.5, and for this reason it remains in the ether (Fr. IIIc) when the pyropheophorbide *a* is extracted. Fraction IIIc was extracted with 22% HCl to remove the pyropheophorbide *b*, and the acid solution (Fr. IIIf) was washed with ether to remove occluded porphyrin contaminants of higher HCl number.

The acid solution of pyropheophorbide *b* (Fr. IIIf) was overlayered with ether and then diluted to approximately 11% acid concentration to drive the pigment back into the solvent (Fr. IIIg). This fraction was then dried *in vacuo* and dissolved in pyridine for pyrolysis. The conditions for the pyrolysis were the same used to form pyropheophorbide *a*, and the techniques for purification and crystallization were the same.

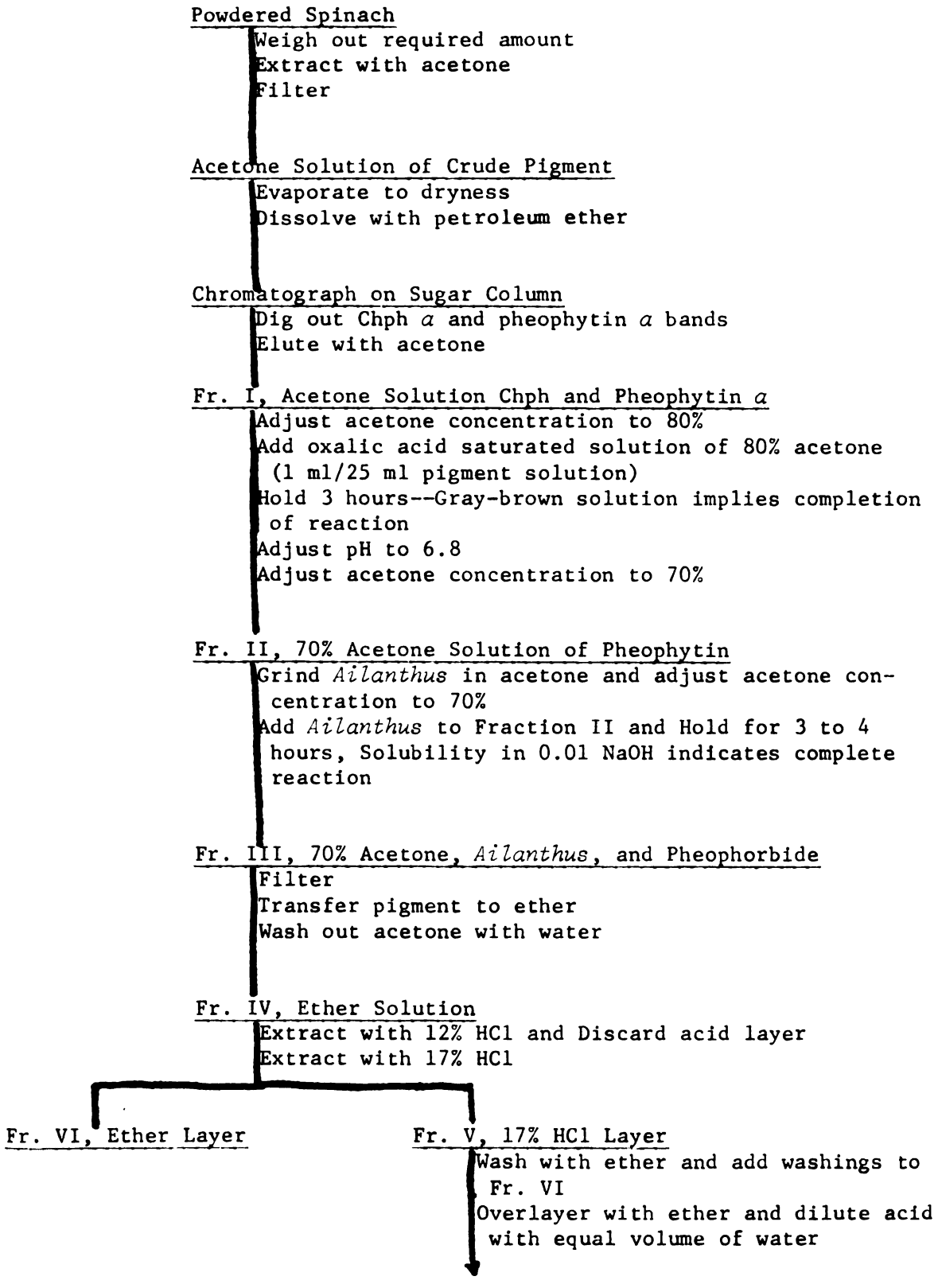
The compound produced was confirmed to be pyropheophorbide *b* by the visible spectrum and the IR data, which had the absorption band of the C_3 aldehyde group at 1663 cm^{-1}. This band was not present in the pyropheophorbide *a* spectra.

Pheophorbide *a*

Pheophorbide *a* was prepared in the following manner: Chlorophyll *a* was isolated from dried spinach by the method of Strain and Svec (107) and dissolved in acetone (Fr. I). Oxalic acid was added to remove the magnesium from the compound (one ml of acid saturated 80% acetone/25 ml of chlorophyll solution), the acetone concentration was adjusted to 70%, and the pH was adjusted to 6.8-7.0. A 70% solution (Fr. II) of ground *Ailanthus* leaves was added, and the dephytylation was allowed to proceed for three to four hours (complete solubility of porphyrin from ether in 0.01 N NaOH was the criterion for conversion to pheophorbide *a*). The pheophorbide (Fr. III) was transferred to ether (Fr. IV) and extracted with acid to fractionate the different products. The 12% acid fraction was discarded while the pigment in the 17% acid, Fr. V, was retransferred to ether (Fr. VI), washed to remove the acid, dried *in vacuo*, taken up in petroleum ether, purified according to the procedure of Holt and Jacobs (54), and collected by crystallization from ether (Fr. VII) by dilution with petroleum ether.

The compound produced by this procedure had the following characteristics of pheophorbide *a*: the HCl number was 15; the visible spectra contained the bands reported by Hashimoto and Tsutsumi (51), Todd and Galston (111), and Stern and Wenderlein (104); the IR spectra contained bands for the C_{10} carbomethoxy group (1741 cm^{-1}), C_7 carboxyl (1710 cm^{-1}), C_9 ketone (1702 cm^{-1}), and C_2 vinyl group (1622 cm^{-1}). There was no phytol absorption, and the fingerprint absorption pattern (absorption bands in the region 1300-650 cm^{-1}) matched the data of Holt and Jacobs (54).

Figure 6. Schematic representation of the methods employed to produce pheophorbide *a*.



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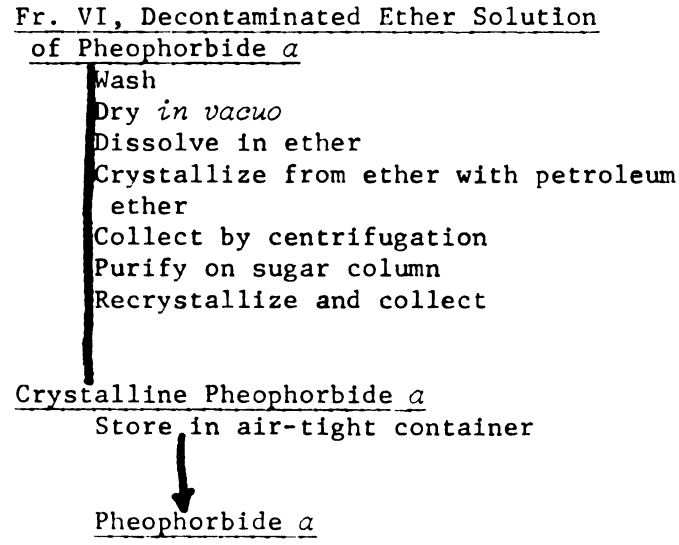


Pheophytin *a*

The pheophytin *a* used in this study was prepared in the following manner: A 100 ml acetone solution of chlorophyll *a* (Fr. I) (prepared in the method of Strain and Svec (107) and at a concentration of 50-100 mg/ml) was treated with a saturated solution of oxalic acid in 80% acetone. One milliliter of acid was added for each 25 ml of pigment solution. After three hours, the pheophytin *a* was transferred to ether and washed five times with distilled water to remove the acetone and acid. Following the washing, the ether solution (Fr. II) was dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The pigment was resuspended in petroleum ether (Fr. II) and allowed to crystallize overnight, cooling the flask with dry ice. The pheophytin *a* crystals were collected by centrifugation and dried *in vacuo* for one hour at 100° C.

Solid pheophytin *a* produced in the above manner exhibited the following characteristic properties: the absorption maxima values agreed

with the published values of Stern and Wenderlein (102) and those of Smith and Benitz (97). The IR spectra matched that reported by Holt and Jacobs (54).

Screening of Foods

One of the goals of this project was to develop a bioassay system to analyze for the presence of phototoxic substances in foods. A preliminary screening of foods was undertaken utilizing a modification of the Hashimoto procedure (51) to assay the liver of two domestic animals. Beef and pork livers were used in this study because these animals are consumers of large quantities of porphyrins from their feeds, and these foods are common meats in the diet of many persons.

Since the photodynamic agents we were seeking were porphyrin in nature, we employed a common extraction method for their isolation. A schematic of this separation procedure is provided in Figure 8. The raw livers were minced and extracted with a 5:1 ether:acetic acid solution. The mixture was vigorously shaken several times until the supernatant became colorless. The combined supernatant was washed repeatedly with distilled water to remove the acetic acid. The ether solution was extracted successively with increasing quantities of hydrochloric acid, and the acid solutions were checked for fluorescence. Five, ten, and seventeen percent solutions of HCl were used in the extraction. Any fraction showing fluorescence and a porphyrin visible spectrum was retransferred to solvent, dried, redissolved, crystallized, and screened in our bioassay system for phototoxic properties.

Figure 7. Schematic representation of methods employed to produce pheophytin *a*.

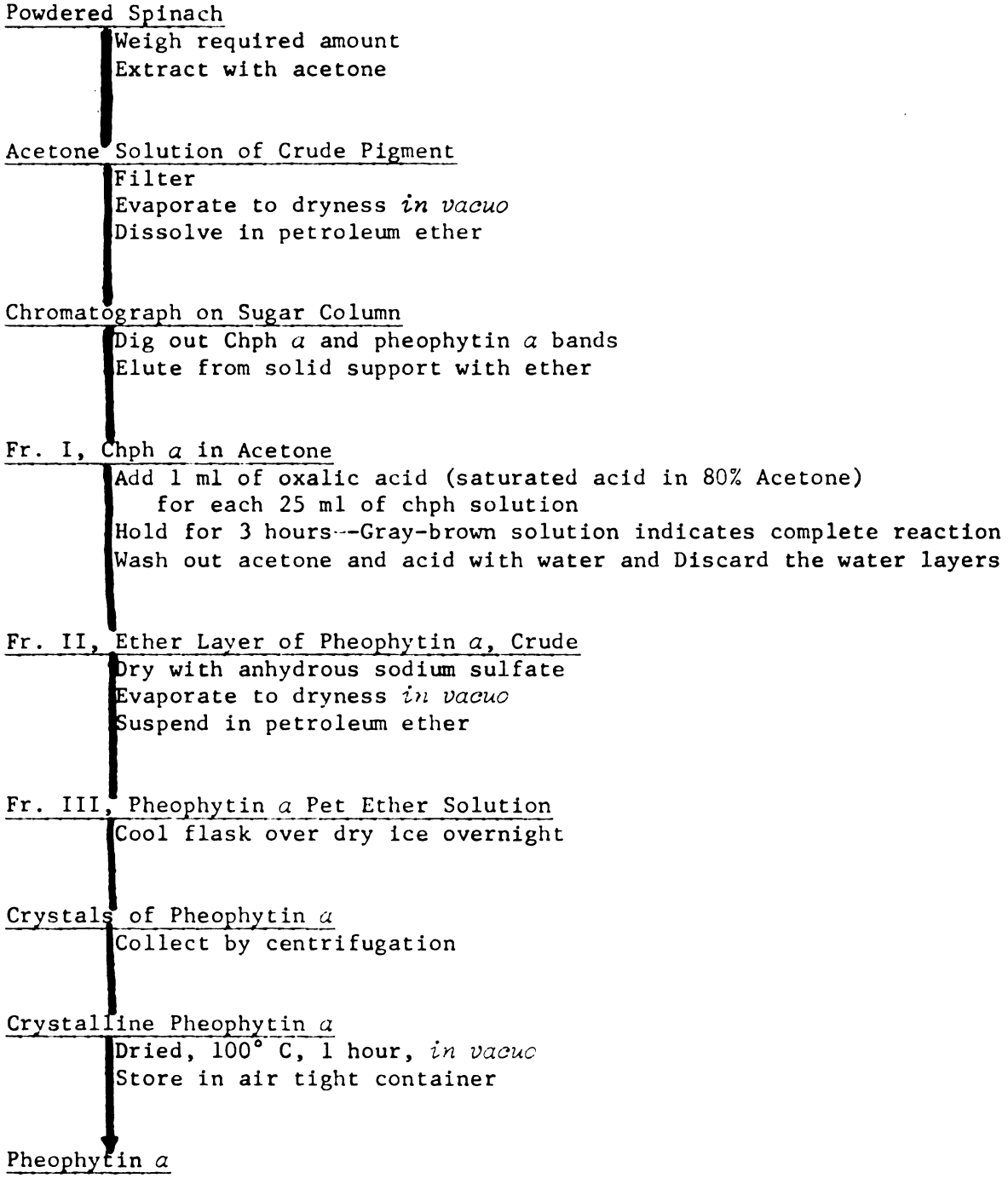
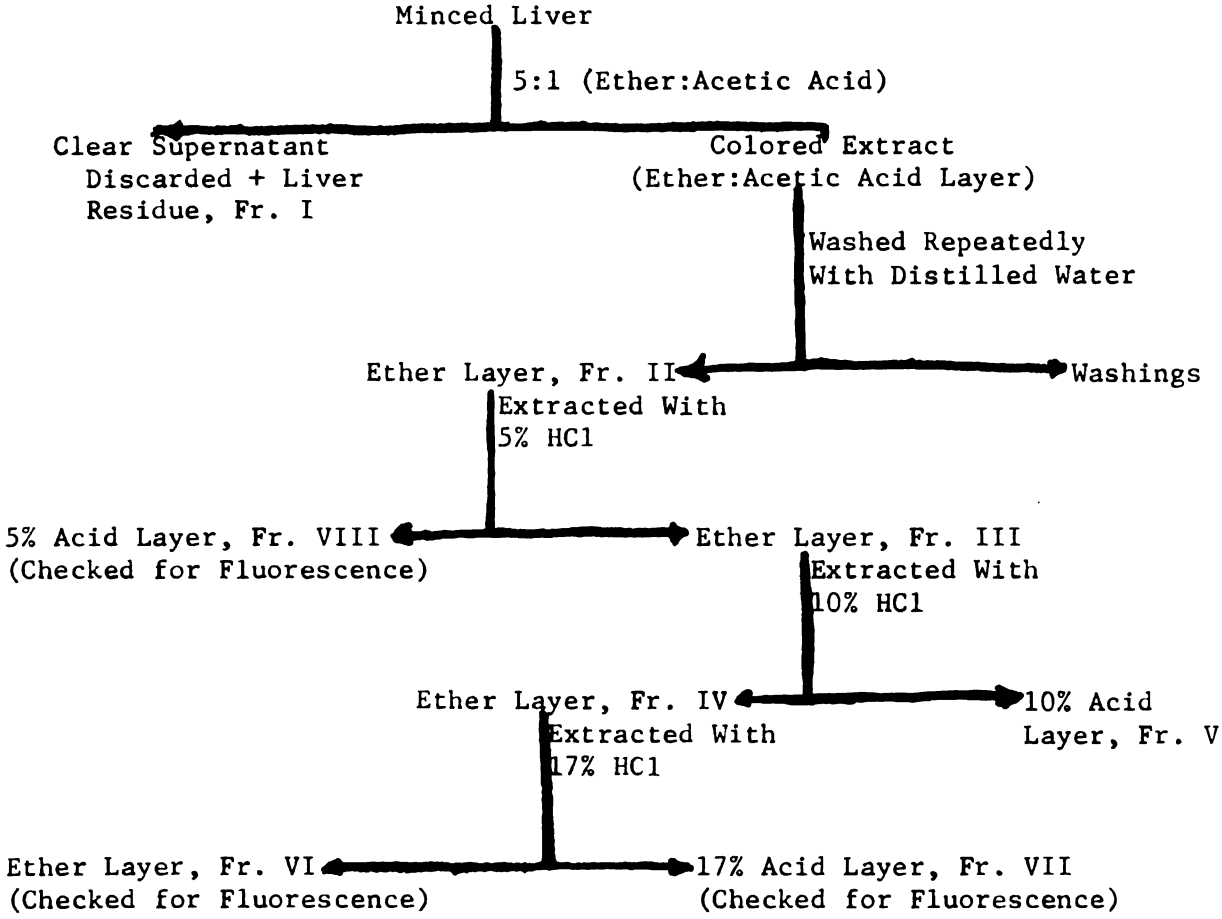


Figure 8. Schematic analysis procedure followed in screening livers for photodynamic agent.



RESULTS

Development of the Assay System

Studies were conducted to develop a bioassay system utilizing albino rats and artificial light to test the photodynamic properties of pyropheophorbide *a* and related derivatives of chlorophyll *a*. The results of these experiments are summarized in Table 1.

The results presented on rats 1 through 7 represent attempts to sensitize the animals with pigment solutions. None of these trials was successful because the crystalline pigment would not form a solution in any of the solvents; vegetable oil, phosphate buffer pH 10.3, and 200 proof ethyl alcohol. The first photosensitized animals were dosed using bread discs with the pigment applied from solutions of oil. This route proved to be the most successful oral method. Palm oil was the best fat for dissolving the pigment and applying it to the bread discs, and a little sugar applied to the impregnated discs made them more desirable to the rats (rats 8 and 9). Rat number 10 was a control and indicated that the bread, oil, acetone, and sugar, used in the preparation of the impregnated wafer, had no phototoxic effect on the rat.

Oral administration of the compound has one serious drawback--the large quantity of pigment required to produce sensitization. A minimum of 6 mg was needed to produce slight sensitization and 10 mg for best results.

Table 1. Summary of the preliminary studies of the photosensitizing properties of pyropheophorbide *a*

Rat No.	Route	Dosage (mg)	Structures Examined: External Ear	Lacrimal Gland	Cornea	Lens	Retina	Photosensitized
1	G,SO	2.0	-	N	N	N	N	-
2	G,SO	4.0	-	N	N	N	N	-
3	G,SO	8.0	-	N	N	N	N	-
4	G,E	0.0	-	N	N	N	N	-
5	G,E	2.0	-	N	N	N	N	-
6	G,E	4.0	-	N	N	N	N	-
7	G,E	8.0	-	N	N	N	N	-
8	O	6.0	+	N	N	N	N	+
9	O	6.0	+	N	N	N	N	+
10	O	0.0	-	N	N	N	N	-
11	O,F	4.0	-	+	N	N	-	-
12	O,F	7.0	+	+	N	N	+	+
13	IP,P[1]	2.0	-	N	N	N	N	-
14	IP,P[1]	4.0	-	N	N	N	N	-
15	IP,C[1]	4.0	-	N	N	N	N	-
16	IV,P,E[2]	1.0	+	+	N	N	+	+
17	IV,D[3]	1.0	+	+	N	N	+	+
18	IV,P,D[4]	0.0	-	N	N	N	-	-
19	IV,P,D[4]	0.5	+	N	N	N	+	+
20	IV,P,D[4]	1.5	+	N	N	N	+	+

Table 1 (cont'd)

Rat No.	Route	Dosage (mg)	Structures Examined: External Ear	Lacrimal Gland	Cornea	Lens	Retina	Photosensitized
21	IV,P,D[4]	2.0	+	+	N	N	+	+
22	IV,P,D[5]	3.0	+	+	N	N	+	+
23	IV,P,D[6]	3.0	+	+	N	N	+	++

B = Buffer, phosphate pH 10.3
C = Carboxy methyl cellulose, CMC
D = Dimethylsulfoxide, DMSO
E = Ethyl alcohol
F = Dr. O. Mickelsen's High Fat Diet
G = Gavage
N = Not examined
IP = Intraperitoneal
IV = Intravenous
O = Orally
P = Propylene glycol
SO = Salad Oil
+ = Lesions present and/or photosensitized
- = No lesions present and/or not photosensitized

1. 0.5 ml
2. P-0.5 ml, E-0.1 ml
3. 0.1 ml
4. P-0.2 ml, D-0.1 ml
5. P-0.3 ml, D-0.1 ml
6. P-0.4 ml, D-0.02 ml

To combat this problem different oral vehicles were utilized. Dr. O. Mickelsen, Foods and Nutrition Department, Michigan State University, provided a high fat diet for our use as an oral vehicle for the compound. The results (rats numbered 11 and 12) indicated that use of this diet did not decrease the amount of pigment required for sensitization.

An attempt was made to decrease the amount of sensitization dosage by injecting the pigment. The results of these experiments are given for rats numbered 13 through 23. Intraperitoneal injections produced no sensitization. Postmortem examination of the animals showed crystalline deposits of the pigment within the peritoneum (Rats 13-15). Intravenous injection in a lateral tail vein of three milligrams of pigment and 0.42 ml of vehicle, 0.4 ml propylene glycol and 0.02 ml DMSO, produced sensitization. This method of administration of the pigment (providing a fifty percent reduction in required pigment) was reconfirmed in numerous studies and adopted as the method for use in the assay system.

The bioassay system, as developed, utilized the following principles, parameters, and techniques:

Artificial light - 20,000 lux at 19" from the light bank.

Test Animal - Albino rat, weight 75-100 g.

Dosage of pigment per rat - 3 mg.

Vehicle - Propylene glycol and DMSO.

Movement - a. If unrestricted, contained in inverted, polystyrene mouse cages.
b. If restricted, anesthetized and exposing only that portion of the anatomy desired

Exposure length - a. 3 hours total; 1.5 hours in two segments, 24 hours between each.
b. Initiated 24 hours postinjection.

Conditions - a. Food and water *ad libitum*
b. Temperature under cages not greater than 28° C
c. Ventilation fan provided to maintain temperature

Signs and Symptoms

Sensitized rats reacted within seconds to exposure to light. Normally, they moved about in an agitated and frantic manner and energetically scratched their exposed areas. After approximately 30 minutes, the animals became subdued, possibly from exhaustion. Most of them then developed a depressed attitude and sat with their feet and tail concealed from the light. Their depression was often interrupted by periods of vigorous activity throughout the remainder of the exposure. Edema and hyperemia developed in the ears, around the eyes, and along the nose. Lacrimation and protrusion of the third eyelid developed around the eye (Figures 9 and 10). The maximum exposure time required to produce these symptoms in the sensitized animals was two hours.

Within twenty-four hours after the first light-exposure, edematous swelling of the face and head was well developed with the eyes protruding. Following the second application of light, the normal deep red color of the eyes faded to a pale pink. Their normal color did not reappear. Within a week, opacities of the lens developed in many rats. The edema of the head and ears disappeared within one week postexposure, and only the eyes appeared abnormal.

Grossly, visible necrosis appeared within thirty-six hours postexposure in those animals most acutely sensitized. The backs of the external ears, the skin across the back of the head, and the skin of the face under the eye to the base of the ear was commonly necrotic. Within ten to fourteen days, the tips of the ears and some of the facial and neck skin sloughed off. A summary of these signs and symptoms appears in Tables 1 and 2.

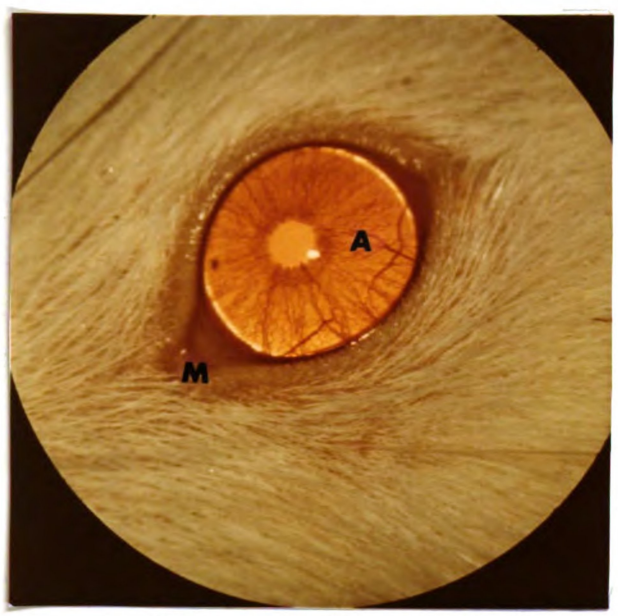


Figure 9. Eye of vehicle control rat two hours after initial exposure of 90 minutes to light. Table 2, Group A. Note normal iris (A), and medial canthus (M). x8.

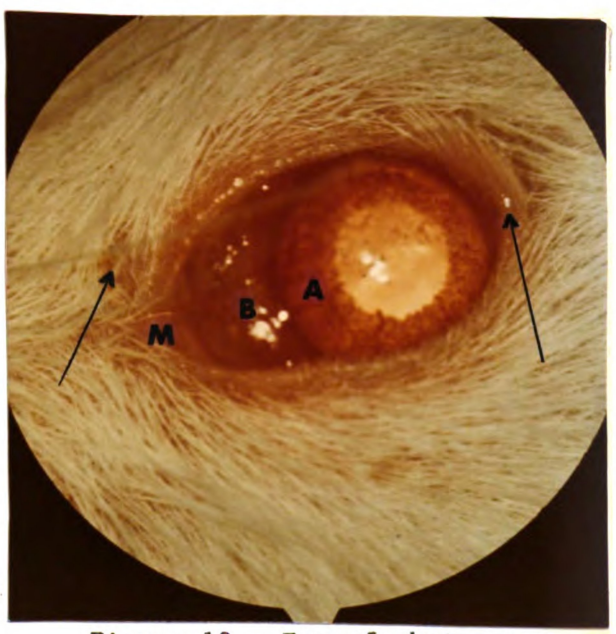


Figure 10. Eye of photosensitized rat two hours after initial exposure of 90 minutes to light. Table 2, Group A,T. Note dilated and hyperemic iris (A), edema and protrusion of nictitating membrane (B), medial canthus (M), and lacrimal secretion on skin around eye (arrows). x8.

Gross and Microscopic Findings

At the time of necropsy, the gross lesions were the same as those seen before death. Pyropheophorbide *a* was used to establish the histological parameters of photosensitization for the assay system. For this reason, the microscopic lesions are summarized as part of the assay of this pigment. The microscopic lesions of the eyes were more extensive than indicated by the clinical observations.

The inflammatory response of the ear was a serofibrinous exudate which obliterated the normal architecture between the epithelium and the cartilage at the center of the ear, doubling its thickness. Distention of the veins and lymphatics also occurred. Sections from inflamed ears, when stained with toluidine blue, showed evidence of mast cell degranulation.

As the summary of eye lesions in Table 2 indicates, there was retinal damage in the eyes of all treated rats, whereas the vehicle control and dark control showed no retinal changes (Figure 11). Twelve hours after exposure the rod cells were basophilic, the pigment epithelium cells were shrunken, and pyknosis, karyolysis, and edema were prominent in the nuclear layers (Figure 12). The changes in the rod cell nuclei were easier to detect in Zenker fixed specimens than in formalin. Serous exudate accumulated in outer plexiform layer and inner nuclear layers. Other histological changes in the retina were not detectable at this stage.

The retinas were extremely edematous after seven days. Fluid accumulated within the retina, retinochosis. The rent developed either in the outer nuclear, the plexiform, or the inner nuclear layer. The retinal layers beyond the inner nuclear layer had mostly disappeared

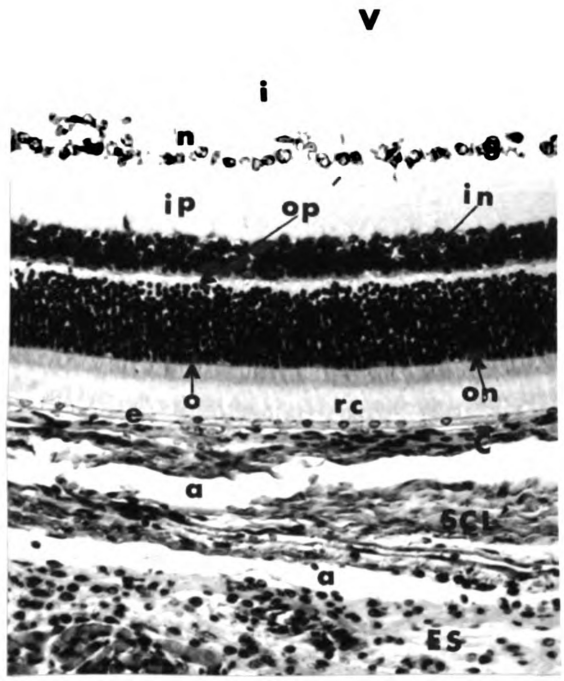


Figure 11. Labeled anatomical features of normal retina and adjacent structure from vehicle control rat. Table 2, Group A. Features which are labeled include: (C) Choroid, (ES) Episcleral tissue with inflammatory cells, (SCL) Sclera, (V) Vitreal space, (a) artifactual tears in ocular coats. Retinal layers from innermost portion to outermost portion: (i) inner limiting membrane--has been pulled from the surface of the retina in processing, (n) nerve fiber layer, (g) ganglion cell layer, (ip) inner plexiform layer, (in) inner nuclear layer, (o) outer limiting membrane--hardly visible, (on) outer nuclear layer, (op) outer plexiform layer, (rc) rod and cone cell layer, and (e) pigment epithelium--pigment lacking in albino rat. Zenker's fixation. Hematoxylin and eosin. x187.

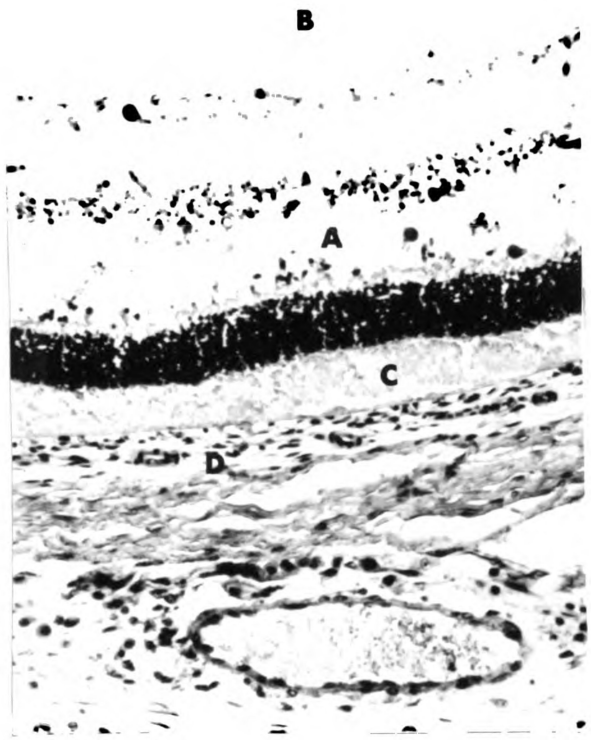


Figure 12. Early retinal degeneration in eye from photosensitized rat. Table 2, Group A,T. Note involvement of all retinal layers with prominent edema and necrosis of inner nuclear layer (A), precipitated material in vitreal space (B), and fragmentation of rod and cone cells (C) (some of the latter is artifact). The choroid (D) is nearly normal. Zenker's fixation. Hematoxylin and eosin. x187.

Table 2. Comparative incidence of microscopic lesions caused by the administration of pyropheophorbide *a* to albino rats

Rat Identification	Dose/Rat (mg)	Killed Post-exposure (days)	External Ear	Lacrimal Gland	Cornea	Lens	Retina
Intravenous Injections							
Group A							
VC*	0	0.5	0/1	1/1	0/1	0/1	0/1
DC**	3	0.5	0/1	0/1	0/1	0/1	0/1
T***	3	0.5	4/4	4/4	3/4	1/4	4/4
Group B							
VC	0	3.0	0/1	1/1	0/1	0/1	0/1
DC	3	3.0	0/1	0/1	0/1	0/1	0/1
T	3	3.0	4/4	4/4	0/4	0/4	4/4
Group C							
VC	0	7.0	0/1	1/1	0/1	0/1	0/1
DC	3	7.0	0/1	0/1	0/1	0/1	0/1
T	3	7.0	4/4	4/4	2/4	2/4	4/4
Group D							
VC	0	14.0	0/1	1/1	0/1	0/1	0/1
DC	3	14.0	0/1	0/1	1/1	0/1	0/1
T	3	14.0	4/4	4/4	1/4	3/4	4/4
Totals							
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
Oral Reconfirmation							
Group E							
VC 1	0	8.0	0/1	1/1	0/1	0/1	0/1
TO 2	10	8.0	1/1	0/1	0/1	0/1	1/1
TO 3	15	a	1/1	0/1	0/1	0/1	1/1

Table 2 (cont'd)

Rat Identification	Dose/Rat (mg)	Killed Post-exposure (days)	External Ear	Lacrimal Gland	Cornea	Lens	Retina
Partial Sensitization							
Group F							
VC	0	7.0	0/1	1/1	0/1	0/1	0/1
T,b	3	7.0	1/1	1/1	0/1	0/1	1/1
T,b	3	c	1/1	1/1	0/1	0/1	1/1

* = Vehicle Control (Rat given vehicle and exposed to the light)
** = Dark Control (Rat dosed with the pigment but not exposed to the light)
*** = Treated (Rats dosed with the pigment and exposed to the light)
TO = Treated Orally (Rat dosed with pigment impregnated on bread discs and exposed to the light)
a = Rat which died 2.5 hours after beginning of exposure
b = Right ear and eye only anatomy of the rat exposed
c = Died 15 minutes after exposure had ended

and mononuclear phagocytes had infiltrated the area (Figures 13, 14, and 15).

Changes were also noted in other structures of the eye. The choroid was infiltrated with a mixture of inflammatory cells in later stages of degeneration. The iris was often not damaged. Lenticular lesions are summarized in Table 2. Cataractous changes were limited to the subcapsular region of the affected lenses. In all instances except one, they were bilateral. Cataracts only occurred in the treated groups. Seven of twenty-four rats, as Table 2 indicates, suffered corneal lesions. Dermatitis and superficial ulceration of the corneal epithelium were the only changes observed.

Extensive damage occurred to the lacrimal gland of all rats exposed to the light. The changes observed in the glands of the treated rats were the same as those in the vehicle control rats. Dark control rats exhibited no damage to their lacrimal glands. The glands were damaged in that portion located adjacent to the posterior aspect of the ocular globe, extending approximately one-half of the thickness of the gland.

The symptoms, clinical and microscopic, observed in the orally treated animals were generally similar to those observed in the rats treated by injection. There was one exception, rat number Gr. E, TO 3. This rat, which had received 15 mg orally, died 15 minutes following a 2.5 hour light exposure.

Application of the Assay System

The bioassay system was then employed to analyze pyropheophorbide *a* and *b*, pheophorbide *a* and pheophytin *a* for phototoxic properties.

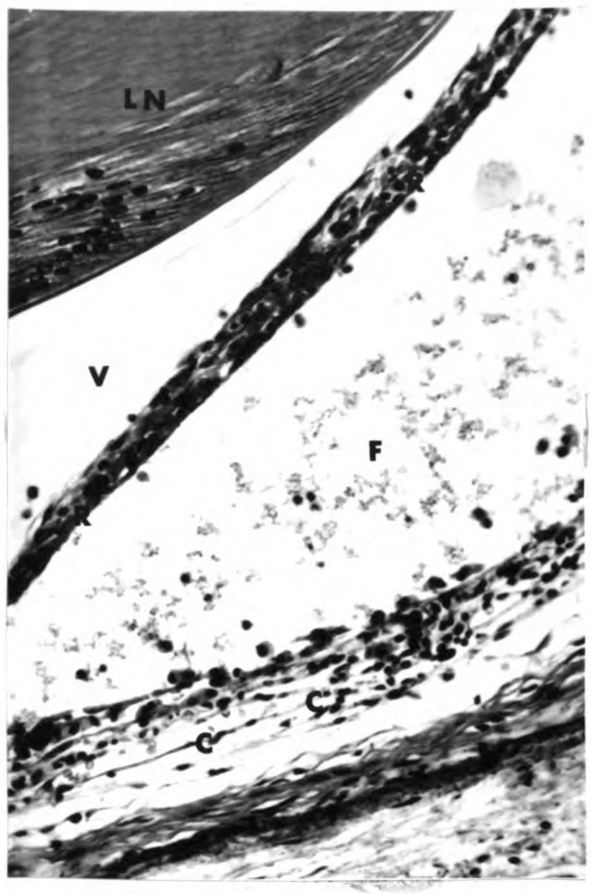


Figure 13. Section through eye of photosensitized rat to show accumulation of fluid in retinal defect with most of vitreal substance displaced. Table 2, Group B,T. Note fluid filled space (F) that elevates retinal remnant (R), and inflammatory cells in choroid (C). (V) is vitreal space, (LN) is part of normal lens. Zenker's fixation. Hematoxylin and eosin. x187.

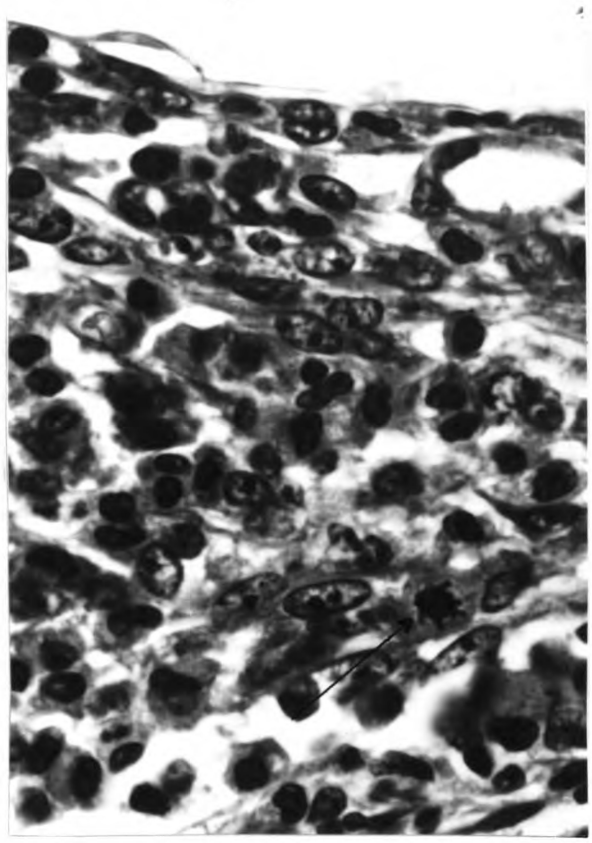


Figure 14. Granulomatous reaction in retina of photosensitized rat. Table 2, Group B,T. Note mitotic figure of proliferating macrophage (arrow). Zenker's fixation. Hematoxylin and eosin. x750.

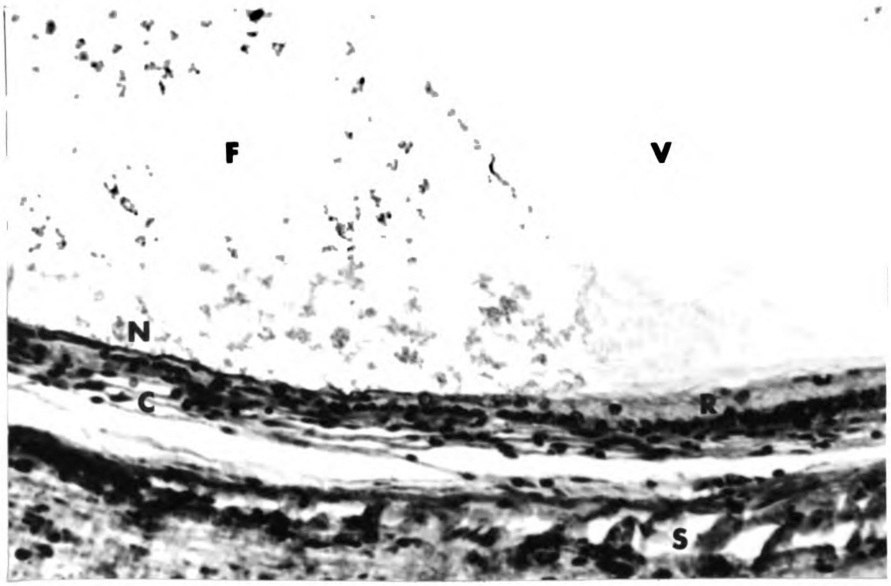


Figure 15. Nearly complete retinal degeneration in eye of photosensitized rat. Table 2, Group C,T. Note remnant of inner retinal layers (R), increased staining of vitreal substance (V)--compared with Figure 9, fluid accumulation which has displaced part of vitreal substance (F), area with no recognizable retina (N), slight infiltration of choroid (C) with inflammatory cells, and distorted sclera (S) which is artifact. Zenker's fixation. Hematoxylin and eosin. x187.

Pyropheophorbide *a*

The phototoxic properties of pyropheophorbide *a* were extensively studied. These data were used to evaluate the histological manifestations of photodynamic action as described in the above sections entitled *Signs and Symptoms* and *Gross and Microscopic Findings*. The intravenous study was designed to last two weeks to offer an opportunity to study the lesions from the incipient stages of retinal lesions through final phases of retinochosis. Retinal damage was observed in all treated animals, and lacrimal gland degeneration was noted in all exposed animals (controls and treated). Retinal degeneration and lacrimal gland damage were not found in the dark control rats (rats given compound and vehicle but not exposed to light). The dark control animals were kept in the animal room where they were in an environment of diffuse light, but the retina was not damaged. No attempt was made to exclude all light from their cages.

Confirmation of the intravenous injection results was performed by an oral assay in adherence to the postulates of Blum regarding proof of photodynamic properties. These tests also showed retinal damage in all treated rats.

A study was also conducted attempting to partially sensitize the treated animals. In this study, the animals were anesthetized, and only parts of them were exposed to the light (right side of the head and face). The animals became photosensitized and developed dry gangrene of the external ear. Their facial skin became deeply necrotic, involving the muscles on the side of the face. The cornea ruptured, and the ocular globe collapsed. All portions of the orbit were involved in primarily a neutrophilic inflammatory process. In contrast to the

Figure 16. Photosensitized rat, Table 2, Group F,T,b. Viewed from right side six 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 17. Photosensitized rat in Figure 16. Viewed from left (unexposed) side, photographed six days after sensitizing exposure. Note normal appearance of eye and ear. Approximately life size.

exposed side of the face, the unexposed (left) side of the face was not affected. The lesions are evident when Figures 16 and 17 are compared.

Pyropheophorbide *b*

The phototoxic properties of pyropheophorbide *b* were assayed in the system. It did not produce sensitivity in the animals under these conditions. The results of this study are summarized in Table 3.

Pheophorbide *a*

Table 3 summarizes the results of the bioassay of pheophorbide *a* in the system. Pheophorbide *a* was shown to be a primary photosensitizing agent. The injected animals showed gross and microscopic symptoms of photosensitization.

Pheophytin *a*

Pheophytin *a* was subjected to bioassay. The data summary in Table 3 indicates that pheophytin *a*, under the conditions of this system, was not a photodynamic agent. The animals exhibited neither gross nor microscopic signs of photosensitization.

Screening of Domestic Foods

A modification of the procedure of Hashimoto and Tsutsumi (51) was used to analyze pork and beef livers for photodynamic agents. While fluorescence was observed in pork fractions V and VIII, analysis of them indicated that none of the phototoxic substances studied in this report was present in these fractions. The livers from both species analyzed under the conditions of this experiment contained no detectable amounts of the photodynamic agents.

Table 3. Comparative incidence of microscopic lesions caused by intravenous injection in rats of pyropheophorbide *b*, pheophorbide *a*, and pheophytin *a*

	Dose/Rat (mg)	Killed Post-exposure (days)	External Ear	Lacrimal Gland	Cornea	Lens	Retina	Photosensitized
Pyropheophorbide *b*								
VC	0	6	0/1	1/1	0/1	0/1	0/1	-
DC	3	6	0/1	0/1	0/1	0/1	0/1	-
TI	3	6	0/2	2/2	0/2	0/2	0/2	-
Pheophorbide *a*								
VC	0	10	0/1	1/1	0/1	0/1	0/1	-
DC	3	10	0/1	0/1	0/1	0/1	0/1	-
TI	3	10	2/2	2/2	0/2	0/2	2/2	+
TO	6	10	2/2	2/2	0/2	0/2	0/2	-
Pheophytin *a*								
VC	0	10	0/1	1/1	0/1	0/1	0/1	-
DC	3	10	0/1	0/1	0/1	0/1	0/1	-
TI	3	10	0/2	2/2	0/2	0/2	0/2	-
TO	6	10	0/2	2/2	0/2	0/2	0/2	-

VC = Vehicle Control (Rat given vehicle and exposed to the light)
DC = Dark Control (Rat given pigment and not exposed to the light)
TI = Rat Treated by Injection and exposed to the light
TO = Rat Treated by Oral administration of the pigment and exposed to the light

Table 4. Summary of the analysis of pork and beef livers for photodynamic agents

Fraction Number	Liver Source		Presence of Pheophorbide *a* and Pyropheophorbide *a*	
	Beef	Pork	Beef	Pork
V	NF	YF	No	No
VI	NF	NF	No	No
VII	NF	NF	No	No
VIII	NF	YF	No	No

Fraction numbers correspond to fraction numbers in separation scheme presented in Figure 8.

NF = No red fluorescence
YF = Red fluorescence observed

DISCUSSION AND CONCLUSIONS

The general syndrome observed in this study implies that pheophorbide *a* and pyropheophorbide *a* are primary photosensitizers, fitting the classification by Clare (25) of photodynamic agents. Clinical signs and microscopic changes of the ear and cornea are almost identical to other reports of photosensitization (1, 8, 15, 24, 25, 28, and 112). From the results of this study it is clear that the rats were photosensitized by intravenous injections and ingestion of pyropheophorbide *a* and injection of pheophorbide *a*.

Pyropheophorbide *a* meets two of the criteria of Blum (15) for being classified as a photodynamic agent, and, within the interpretation of these postulates by Clare (25), it can be considered a photodynamic agent. Pheophorbide *a* also meets the criteria. The fact that oral administration of the latter compound did not produce photosensitization can be attributed to failure of the animal to absorb the material from the digestive tract or to the feeding of insufficient material (only the minimum oral dose was fed). The changes in the eyes and ears of the injected animals clearly showed evidence of a phototoxic response.

Intraocular tissue lesions and lacrimal gland lesions were reported by Mathews (as cited in 8) in his studies of rat photosensitization. Several reports were found in the literature which associate intraocular lesions with photosensitization or photosensitizing agents. A direct cause and effect relationship between a photodynamic agent and these lesions was not established.

Results of the oral studies (Tables 1 and 2) show that 6-7 mg of pigment were required as the threshold dose of pigment which induced sensitization. These findings agree with the 7 mg level cited by Clare (26) and Tsutsumi and Hashimoto (112) as the minimum oral dose for sensitizing rats. The absence of retinal damage in vehicle and dark control rats and in the unexposed eyes of rats in Group F, Table 2, is evidence that the light, the agent, or the vehicle alone were not the source of the retinal damage.

The retinal damage observed in this study differs in several important aspects from the light induced retinal lesions reported by Noell and others (76). They reported that a continuous exposure to light for 24 hours was required to produce irreversible retinal changes when the animals were exposed at normal body temperature. Under these conditions histological changes were not present until 4-6 weeks after exposure. They were never successful in completely destroying the retina. Portions of the inner nuclear layer and layers internal to it always remained. Dantzker and Gerstein (32) observed the same histological changes that Noell and others (76) had reported.

The light source we used was more intense than that used by Noell and others (76) and Dantzker and Gerstein (32). Although the more severe retinal changes of the present study might be caused by the greater light intensity, the absence of lesions in the eyes of the vehicle control rats tends to refute such a suggestion.

The work of Pierpaoli and Santamaria (as cited in 88) on light induced lesions in calf retina tends to support the work of Noell and others (76) and Dantzker and Gerstein (32), but it is not helpful in understanding the retinal changes produced in this study. Due to the

rapid elimination of eosin from the body, the report by Walkowicz (122) of retinal changes attributed to this compound is difficult to interpret. The seven day period between injection and exposure raises the question of what amount of this rapidly eliminated compound would be present to induce the lesions on exposure.

Jaffe (55) and Barnes and Boshoff (9) have reported clinical observation of retinal lesions in porphyric patients without histological confirmation. The rate of incidence in the Barnes and Boshoff report was greater than fifty percent of the porphyria cases, illustrating the need for further study of retinal damage caused by photodynamic action.

Explaining why retinal damage occurs in this study while it has not been reported in the many other photosensitizations of rats and other animals, at this time, can only be conjecture. Following are some possible suggestions:

1. Pathologists often disregard the eye; and therefore, the retinal lesions are overlooked.
2. Located in deeper tissues, the photodynamic agent does not receive sufficient energy of the required wavelength to excite the compound.
3. Distribution of the photodynamic agent is at the wrong location within ocular tissues to receive the radiant energy.
4. Because the albino rat lacks pigment in the pigment epithelium layer, there is greater penetration, making their eye unusually susceptible to photodynamic damage.
5. Photodynamic action is not responsible for the retinal damage. Relying on the evidence at hand, this alternative cannot be entirely eliminated.

All rats exposed to light, regardless of other factors, had lesions of the lacrimal gland. Those glands of rats not exposed to light were normal. The histological changes in the lacrimal gland were consistent with photosensitization. Reports could not be found in the literature to support this observation.

The results of the analysis of beef and pork livers revealed that the primary photosensitizers, pyropheophorbide *a* and pheophorbide *a*, were not extracted from the livers of these animals. Plausible explanations as to why they were not found include:

1. Cows and pigs do not metabolize chlorophyll to these derivatives; therefore, they could not be found in the livers.
2. The porphyrins are not absorbed from the digestive tract.
3. The extraction system, a classical for extraction of porphyrins, was not adequate for extraction of these compounds from the liver tissue.
4. Pyropheophorbide *a* and pheophorbide *a*, if they were present in this tissue, were there in such minute amounts that they were not detectable among co-extractants.

From these results it can be concluded that primary photosensitizers of the pyropheophorbide *a* and pheophorbide *a* type were not present in the pork and beef livers in sufficient quantities, if at all, to be a health problem to persons eating them. Unlike the abalone whose sources of food are algae and seaweed, which are high in chlorophyll, and whose toxicity is correlated to the algae blooms, pigs and cows ingest enough other materials so that the porphyrin content per unit of food intake would not be sufficient to produce livers with levels of agents high enough to induce phototoxicity.

The chlorophyll content of edible green vegetables is too low to be a photosensitizing hazard at the levels of dietary intake. The bulk of these vegetables required to produce 6-7 mg of sensitizing agent would be so filling that a person could not ingest enough of the food to get this amount of sensitizer into his system.

In the case of persons suffering from a damaged or diseased liver, green vegetables could be toxic, causing the hepatogenous or the secondary type of photosensitization. The liver under these

circumstances fails to detoxify the porphyrins from the body allowing them to enter the circulatory system reaching the epidermis of the skin and on exposure to light producing a phototoxic response in a manner analogous to *geeldikkop* in animals. Eating green vegetables might result in photosensitization from pyropheophorbide *a* or pheophorbide *a* if a person had a damaged liver.

RECOMMENDATIONS

Primary photosensitizers, pyropheophorbide *a* and pheophorbide *a*, used in this assay system could provide a model system for mechanistic studies of the photosensitization process, following the procedures of Bellin (10, 11, and 12). The action spectrum should be derived, and the kinetics of the *in vitro* and *in vivo* processes should be studied for these systems.

Early retinal changes are not localized. The rod cells and the rod cell nuclei exhibit the first observable changes. After injury, the progress of degeneration is rapid. A study of the progressive changes using the microscopic techniques of Allison (1 and 2) and Slater (94) might provide evidence for cause and effect relationship between sensitizer and intraocular lesions. A radioactive tracer in the agent and autoradiography could also provide information on this relationship.

Pyropheophorbide *b* and pheophytin *a* were not primary photosensitizing agents under the conditions of this system. Experiments should be designed to determine the role of differences in molecular structure in causing photosensitization.

Although photodynamic agents related to chlorophyll could not be found in the livers of domestic animals, the value of this assay system is not lessened. With the current trend toward foods produced from algae, and the future possibility of foods formulated from other

plant proteins, the importance of assaying them for phototoxic substances must not be overlooked.

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