Agric. Biol. Chem., **46** (9), 2183～2193, 1982

Isolation of 10-Hydroxypheophorbide *a* as a Photosensitizing Pigment from Alcohol-treated *Chlorella* Cells

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Received May 1, 1981

A new pigment causing intense photosensitivity in rats was isolated from alcohol-treated *Chlorella* cells and identified as 10-hydroxypheophorbide *a* by chemical analysis, chromatography, and visible, infrared, nuclear magnetic resonance and mass spectroscopy methods.

When rats administered orally with this pigment were exposed immediately to the visible light, signs of the intense photosensitivity including death occurred after a few hours of photoirradiation. The photosensitizing activity of this pigment in rats was markedly higher than those of pheophorbide *a* from *Chlorella* cells and pyropheophorbide *a* from pickled greens. Chlorophylls *a* and *b*, pheophytin *a*, and methyl and ethyl pheophorbides *a* were inactive under the same experimental conditions

Microalgae containing chlorophylls (Chl's), such as *Chlorella, Scenedesmus* and *Spirulina*, have been studied for many years for utilization as future food resources, since they produce proteins and various vitamins at high efficiency under artificial mass culture conditions.[1] Recently, because of the nutritional and medical characteristics of these algae, they have come to be utilized practically as a health food in Japan.

In 1977, there was an outbreak of photosensitivity dermatitis among persons who had taken a certain brand of *Chlorella* tablets. Tamura *et al.*[2] reported that this peculiar dermatitis was a typical dietary photosensitivity disease and the causative agent was postulated to be pheophorbide *a* (Phd) derived from chlorophyll *a* (Chl) during ethanol-treatment of *Chlorella* cells with a high chlorophyllase activity.

During the course of our studies on the photosensitivity caused by *Chlorella* cells, we found a new photosensitizing pigment causing more intense lesion in rats than Phd in ethanol-treated *Chlorella* cells. The present paper deals with the isolation and the identification of this pigment and its effect on rats, comparing with some chlorophyll derivatives. A photosensitizing pigment from pickled greens[3,4] was also investigated.

MATERIALS AND METHODS

Isolation and purification of the photosensitizing pigments The strain used was *Chlorella regularis* S-50.[1] The cells grown under heterotrophic pure culture conditions[5] were harvested by centrifugation and washed with water A part of the packed cells was suspended in water (cell conc. about 500 ml/liter), heated with a plate heater (type; SHX-C2HR-2000, Iwai Kikai Kogyo Co.) at 130°C for 15 sec and then dried with a spray drier (type; NIRO atomizer mobile minor, Ashizawa NIRO Co.). This was designated as Chlorella powder (I). One kg of another part of the fresh packed cells containing about 3.8 g of Chl's was suspended in 3 liters of 60% aqueous ethanol and bubbled with air at 36°C for 2 days. A part of the cell suspension was dried under vacuum and the powder was named as Chlorella powder (II). Isolation of the photosensitizing pigments from the cell suspension in aqueous ethanol was performed according to the method of Tamura *et al.*[2] with some modifications. By centrifugation of the cell suspension, the green extract was separated from the cell residue, which was further extracted 3 times with 3 liters each of methanol. The combined green extract (about 10 liters) was concentrated under vacuum to half its volume and stirred vigorously with 2 liters of chloroform. After washing with water, the chloroform layer which separated from the aqueous layer was evaporated to

dryness under vacuum. The dried material (F-I, alcohol extract) was dissolved in 1 liter of ethyl ether and the solution was treated 4 times with 17% HCl solution. The pigments were partitioned off between the two liquid layers. The ether layer was washed with water and evaporated to dryness under vacuum (F-II, ether fraction). The 17% HCl fraction was diluted with water to a 5% HCl solution and the pigments were retransferred to ethyl ether. The ether layer was washed with water and evaporated to a small volume (F-III, 17% HCl-2nd ether fraction) under vacuum When the 5% HCl layer was diluted with water to a 1% HCl solution and extracted with ethyl ether, small amounts of pigments were transferred to the ethereal layer, which was then evaporated to dryness under vacuum (F-IV, 5% HCl fraction).

The Chlorella F-III fraction was streaked or spotted on a thin layer plate of silicagel (20 × 20 cm, 0.25 mm for preparation, 20 × 5 cm, 0.25 mm for analysis, Merck KG 60) and developed with a solvent mixture of benzene, ethyl acetate, ethanol and *n*-propanol (16 : 4 : 1 : 1) at 4°C in the dark. The colored bands separated on the plate were scraped up and individually extracted with methanol. Each methanolic extract was mixed with 2 volumes of chloroform and the resulting solution was washed with water. The pigment solution (in chloroform) was concentrated under vacuum and 2 volumes of petroleum ether added to the concentrated solution. The solution was allowed to stand in an ice box for 3 hr. The black crystals formed were collected and dried at 100°C for 1 hr under vacuum.

The photosensitizing pigments contained in pickled greens (*Brassica juncea*) were also isolated by the above procedure. The fraction of pickled greens corresponding to Chlorella F-III was designated as pickle F-III.

Preparation of methyl esters of the isolated pigments. Methylation of the pigments isolated by thin layer chromatography was carried out according to Pennington's method with some modifications.[6] Each pigment (100 mg) was dissolved in about 20 ml of 2% HCl-methanol and boiled under reflux for 1 hr. After cooling, the pigment was transferred to ethyl ether, washed with water and evaporated to a small volume. The pigment solution was applied to similar thin layer chromatography as previously described, to separate the esterified from the unesterified pigment.

Ethyl esters of the pigments were also prepared by the same procedure as described above using ethanol instead of methanol.

Preparation of chlorophylls a and b, and pheophytin a. The Chl's were isolated from fresh *Chlorella* cells. Before extraction, the *Chlorella* cell suspension in water was heated with a plate heater at 130°C for 15 sec. Chl's *a* and *b* were extracted with methanol-petroleum ether and were further purified by the chromatographic method according to Strain *et al.*[7] Pheophytin *a* was prepared by briefly shaking the petroleum ether solution of Chl with dilute HCl and was purified by the method of Perkins and Roberts.[8]

Spectroscopy. Absorption spectra in the visible and ultraviolet regions were measured with a Hitachi 200-10 spectrophotometer and those in the infrared region with a Jasco A-102 spectrophotometer. Mass spectra were obtained using direct inlet systems with a Shimadzu LKB 9000 under the following conditions: ionizing energy, 70 eV; ionizing current, 60 μA; source temperature, up to 270°C. Nuclear magnetic resonance (NMR) spectra were measured with a JNM FX-100 instrument at room temperature. Chemical shifts in $CDCl_3$ were given in units of δ (ppm), from tetramethylsilane as the internal standard.

Photosensitizing activities of the isolated pigments.

a) *Animal and diets.* Albino rats of Wistar strain (about 100 g) were used, housed in threes. The basal diet used was Diet M of Oriental Yeast Co Chlorella powder (I and II) was added to the basal diet at 50% and given orally to the rats. The isolated pigment or each fraction (Chlorella F-I, II, III, IV, and Pickle F-III) was dissolved or dispersed in 0.5 ml of distilled water and administered orally to the rats through a stomach tube. Body weight and food consumption were recorded daily. Water was available at all times.

b) *Photoirradiation.* After administration of the isolated pigment or each fraction termed F-I to F-IV, the rats were irradiated immediately from a distance of 50 cm through a water layer with Toshiba 500W tungsten lamps. The emission range was from 400 to 1000 nm and the light intensity was about 100 mW/cm^2 on the back of the rat. In cases of a single administration, the rats were irradiated for 8 hr (10 : 00 a.m. ~ 6 : 00 p.m.) daily. In cases of daily administration, the rats were irradiated 8 hr daily for 2 weeks from the start, and in a subsequent period, 7 hr (10 00 a.m. ~ 5 : 00 p.m.) daily, for 5 days in a week. The food cup was removed during irradiation to avoid shading the rats. The temperature was kept at 25°C.

RESULTS

Isolation of the photosensitizing pigments

When rats fed for 4 days on diets containing 50% of the Chlorella powder (II) (alcohol-treated *Chlorella* cells) were exposed to light, they became immediately restless, then lethargic, and finally about 6 hr later, died from a disease resembling acute circulatory insufficiency as described later. However, rats fed on the Chlorella (II) diets in the dark, and the basal diets or diets containing 50% of the

TABLE I. PHOTOSENSITIZING ACTIVITIES OF THE SEPARATED FRACTIONS IN RATS IN A SINGLE ORAL DOSAGE

Fraction	Dose (mg/kg)	Photosensitivity response	
		No. of deaths / No. of rats irradiated	No. of rats with skin lesions* / No. of survivors
Alcohol extract (F-I)	200	2/3	0/1
Ether fraction (F-II)	500	0/3	0/3
17% HCl-2nd ether fraction (Chlorella F-III)	200	2/4	2/2
5% HCl fraction (F-IV)	200	0/3	0/3
17% HCl-2nd ether fraction (Pickle F-III)	200	3/3	—

* The skin lesions refer only to those externally apparent in the rats.

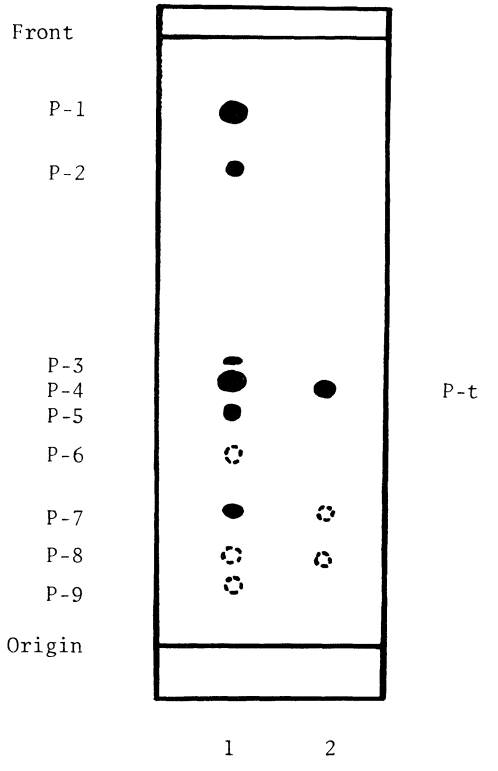

FIG. 1. Thin Layer Chromatography of 17% HCl-2nd Ether Fractions from Alcohol-treated *Chlorella* Cells and Pickled Greens.

Thin layer, silica gel plate (Merck, KG 60); solvent system, benzene–ethyl acetate–ethanol–*n*-propanol (16:4:1:1); samples, (1) 17% HCl-2nd ether fraction from *Chlorella* cells (Chlorella F-III); (2) 17% HCl-2nd fraction from picked greens (pickle F-III).

Chlorella powder (I) (130°C heated *Chlorella* cells) in the light, did not show any apparent symptoms.

The photosensitizing activities of the various fractions observed in rats after a single oral dosage are shown in Table I. F-I (alcohol extract) and F-III (17% HCl-2nd ether fraction) from Chlorella and pickled greens showed the photosensitizing effect in a single dosage of 200 mg/kg of rats.

On the other hand, the F-II (ether fraction) and F-IV (5% HCl fraction) were inactive in a dosage of 200 mg/kg.

To isolate the photosensitizing pigments, F-III of Chlorella and pickled greens were applied to thin layer chromatography (Fig. 1). Chlorella F-III was separated into four major pigments (P-1, P-4, P-5, P-7) and five minor ones (P-2, P-3, P-6, P-8, P-9). Pickle F-III consisted of one major (P-t) and two minor pigments.

As will be shown later, among the purified pigments, P-4 (*Rf* 0.39) and P-5 (*Rf* 0.34) from Chlorella and P-t (*Rf* 0.38) from pickled greens were found to cause the intense photosensitivity in rats in a single administration of 200 mg/kg of rats. The other pigments were less active. The yields of P-4 and P-5 prepared from 1 kg of *Chlorella* packed cells containing 3.8 g of cellular Chl's were 670 mg and 470 mg, respectively, and that of P-t from 100 g of dried

pickled greens was 650 mg.

Spectra of the photosensitizing pigments

P-4, P-5, and P-t were converted into methyl esters (termed methyl P-4, methyl P-5 and methyl P-t, respectively) and subjected to the following analysis. Ethyl P-4 was also prepared.

a) *Absorption spectra in the visible and ultraviolet regions.* Absorption spectra of P-4, P-5 and P-t, and their methyl esters in ethyl ether are shown in Fig. 2. Since crystals of P-4, P-5 and P-t were dissolved directly in ethyl ether to only a limited extent, they were once dissolved in a 17% HCl solution, and diluted with 4 volumes of water. An equal volume of ethyl ether was added, and the pigments were transferred to the ethyl ether by vigorous shaking. The ether layer was exhaustively washed with water to remove the HCl.

The spectra of P-4, P-t, methyl P-4 and methyl P-t are essentially identical with that of pheophytin *a*.[9,10] However, slight differences are observed between P-5, methyl P-5 and the other pigments in the ratio of the absorbance at the blue and the red peaks (E_{667}/E_{409}; P-5 and methyl P-5, 1.92～1.94; the others 2.05～2.10), the shoulder pattern between 300 and 400 nm and a small peak at 533 nm.

b) *Infrared spectra.* As shown in Fig. 3, the infrared spectra of methyl esters of P-4, P-5 and P-t in $CHCl_3$ are almost identical to that of methyl pheophorbide *a* (methyl Phd) reported by Katz *et al.*[11] The methyl esters of P-4 and P-5 have clear absorptions of ester carbonyl at 1735 cm^{-1} and of ketone carbonyl at 1690 cm^{-1}, while the former for methyl P-t shows much lower intensity than that for the latter. A band at 3530 cm^{-1} was observed only in the spectrum of methyl P-5 and assigned to a hydroxy group.

c) *NMR spectra.* NMR spectral data of the methyl esters of P-4, P-5 and P-t are summarized in Table II, together with those of methyl Phd and methyl pyropheophorbide *a* (methyl Pyrophd) cited from Pennington *et al.*[6] The nomenclature and proton designations of the chlorophyll derivatives are also

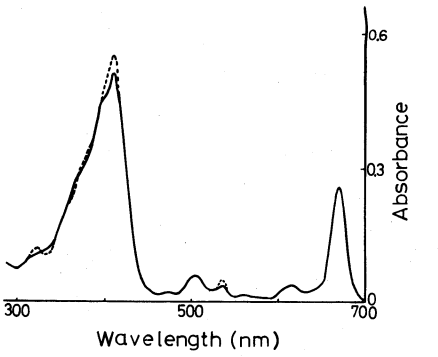

FIG. 2. UV and Visible Absorption Spectra of the Photosensitizing Pigments and their Methyl Esters in Ethyl Ether.

------, P-4, methyl P-4, P-t, methyl P-t; ——, P-5, methyl P-5.

The absorbances of each pigment were adjusted so as to coincide at the red peak.

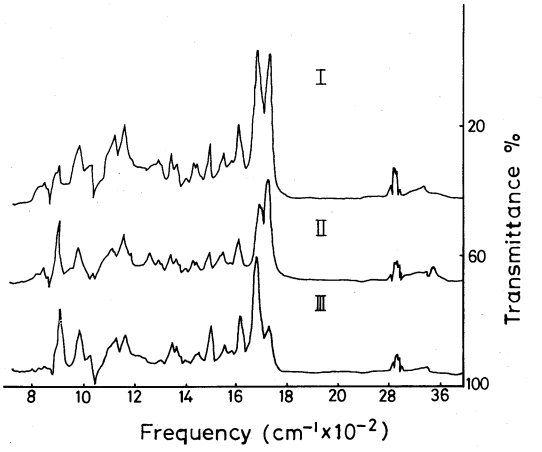

FIG. 3. IR Spectra of Methyl Esters of the Photosensitizing Pigments.

Solvent, $CHCl_3$; concentration, 2% (w/v); I, methyl P-4; II, methyl P-5; III, methyl P-t.

given in Fig. 4. The chemical shifts of methyl P-4 and methyl P-t were essentially identical with those of methyl Phd and methyl Pyrophd, respectively. The methyl regions of methyl P-4 and methyl P-5 showed the same number of methyl and methoxy groups and the low field methine regions were likewise similar.

The most prominent difference was the absence of the C-10 proton resonance at 6.25 ppm in the spectrum of methyl P-5, and its

TABLE II. NMR ASSIGNMENTS OF METHYL ESTERS OF PHOTOSENSITIZING PIGMENTS

Chemical shifts (δ, ppm): solvent, $CDCl_3$; concentration, 0.004% (w/v).

Proton	Methyl P-4	Methyl P-5	Methyl P-t	Methyl* pheophorbide *a*	Methyl** pyropheophorbide *a*
β	9.42	9.57	9.44	9.32	9.32
α	9.26	9.43	9.34	9.15	9.20
δ	8.53	8.61	8.54	8.50	8.50
2	7.91	7.99	7.97	7.85	
2′	6.22	6.28	6.26	6.12	
2″	6.12	6.18	6.15	6.04	
10	6.25	—	5.17	6.22	5.13
10′	—	5.33	—	—	—
8	4.47	4.73	4.47	4.40	4.42
7	4.21	4.47	4.29	4.13	4.23
11	3.88	3.71	—	3.88	—
5	3.65	3.64	3.64	3.62	3.58
12	3.57	3.56	3.61	3.57	3.58
4				3.48	
1	3.36	3.41	3.39	3.32	3.35
3	3.13	3.23	3.20	3.05	3.13
7′7″	2.2∼2.5	2.1∼2.4	2.2∼2.6	∼2.45	
8′	1.81	1.74	1.81	1.82	1.72
4′	1.68	1.65	1.67	1.60	1.55
13				0.50	∼−1.85
14	−1.67	−1.72	−1.71	−1.75	

*, ** Data from Pennington *et al.*[6]

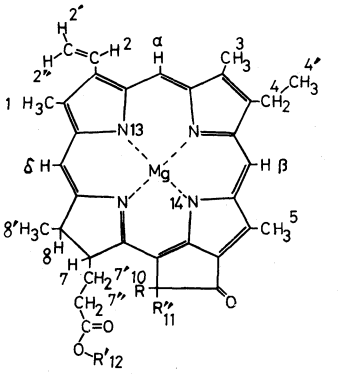

FIG. 4. Nomenclature and Proton Deisgnations of Chlorophyll *a* Derivatives.

	Mg*	R	R′	R″
Chlorophyll *a*	+	H	Phytyl	$COOCH_3$
Chlorophyllide *a*	+	H	H	$COOCH_3$
Pheophytin *a*	−	H	Phytyl	$COOCH_3$
Pheophorbide *a*	−	H	H	$COOCH_3$
Methyl pheophorbide *a*	−	H	Methyl	$COOCH_3$
10-OH-pheophorbide *a*	−	OH	H	$COOCH_3$
10-OH-methyl pheophorbide *a*	−	OH	Methyl	$COOCH_3$
Pyropheophorbide *a*	−	H	H	H
Methyl pyropheophorbide *a*	−	H	Methyl	H

* +, Mg present; −, Mg replaced by 2H (proton Nos. 13, 14).

replacement by a somewhat broader peak at 5.33 ppm, which was assigned to the proton of a hydroxy group. The hydrogen giving rise to this resonance was instantaneously exchanged by the addition of D_2O, unlike the C-10 proton, which requires hours for a complete exchange.[12] The resonances at 3.88 ppm in methyl P-4 and at 3.71 ppm in methyl P-5 arose from the 11-carboxymethyl protons. This 11-carboxymethyl proton in methyl P-t was absent and identical to that of the methyl Pyrophd in reference, giving strong support to the idea that the carbomethoxyl group was eliminated in this compound.

The results for the NMR spectra confirm unequivocally that methyl P-4, methyl P-5 and methyl P-t are methyl Phd, 10-hydroxymethyl pheophorbide *a* (methyl OH-Phd) and methyl Pyrophd, respectively.

d) *Mass spectra.* The mass spectra of three purified pigments and their molecular weights, which were obtained from the molecular ion peak (M^+), are shown in Fig. 5 and Table III. The calculated molecular weights of the pre-

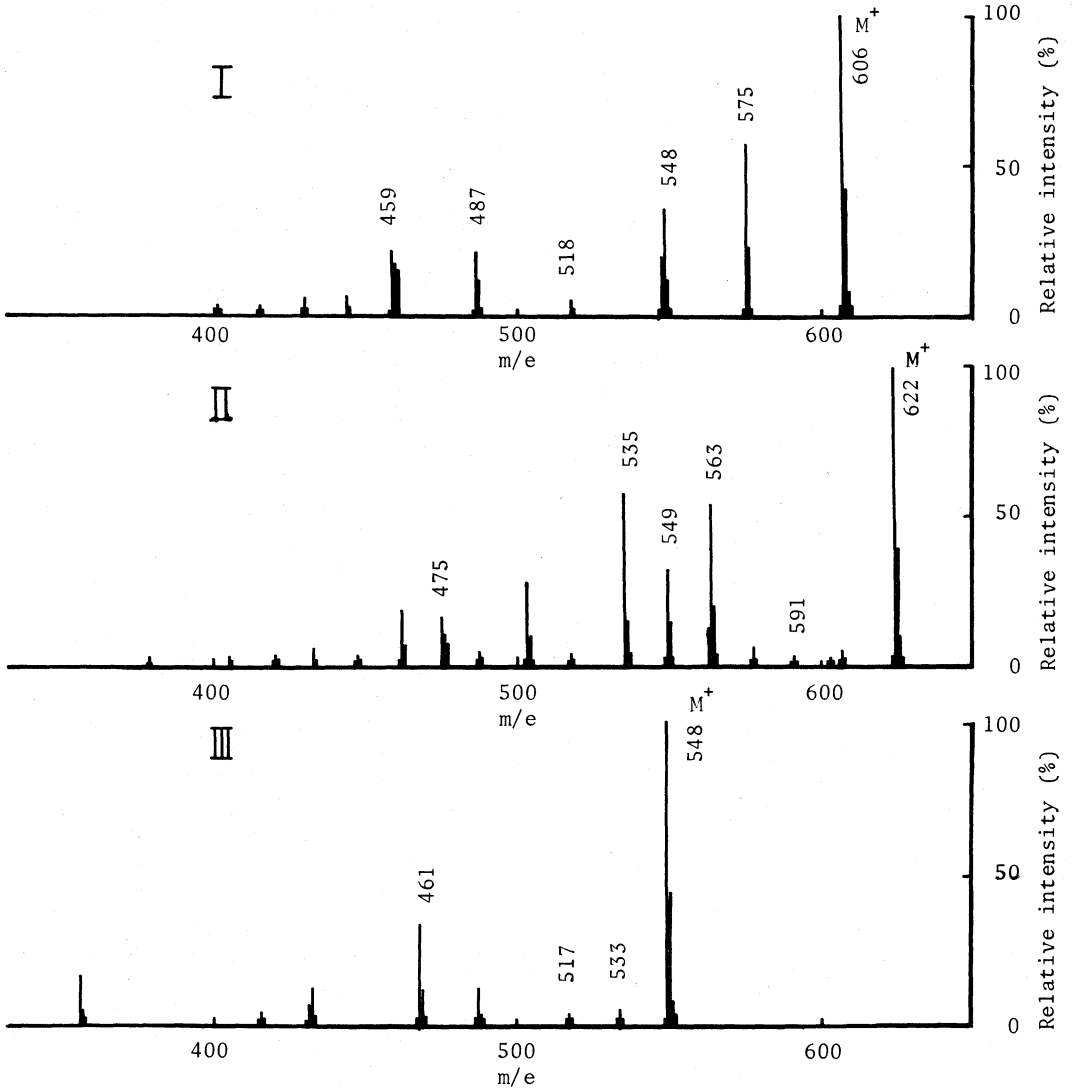

FIG. 5. Low Resolution Mass Spectra of Methyl Esters of the Photosensitizing Pigments.
I, methyl P-4; II, methyl P-5; III, methyl P-t.

TABLE III. MOLECULAR ION PEAKS OF METHYL ESTERS OF PHOTOSENSITIZING PIGMENTS AND MOLECULAR WEIGHTS OF THE CORRESPONDING COMPOUNDS

Compound	Molecular ion peak M^+ (m/z)	Presumed compound	Calculated molecular weight
Methyl P-4	606	Methyl pheophorbide *a* $C_{36}H_{38}O_5N_4$	606.69
Methyl P-5	622	10-Hydroxymethyl pheophorbide *a* $C_{36}H_{38}O_6N_4$	622.73
Methyl P-t	548	Methyl pyropheophorbide *a* $C_{34}H_{36}O_3N_4$	548.66

sumed compounds corresponding to these pigments are also shown in the table for reference. The analytical molecular weights and the types of fragmentations of methyl P-4, methyl P-5 and methyl P-t are exactly equivalent to the respective calculated values of the presumed ones.

Identification of the photosensitizing pigments

The HCl number of P-4, P-5 and P-t were determined to be 15, identical to that of pheophorbide *a*. The Molisch phase tests of P-4 and methyl P-4 were positive, indicating the presence of the activated hydrogen at C-10, but those of P-5, methyl P-5, P-t and methyl P-t were negative, indicating its absence.

All the above observations support the conclusion that P-4, P-5 and P-t are pheophorbide *a*, 10-hydroxypheophorbide *a* (OH-Phd) and pyropheophorbide *a* (Pyrophd), respectively. The molar absorption ocefficients of these pigments at the red max. were Phd, 47,200; OH-Phd, 50,200; Pyrophd, 46,200 $M^{-1}\cdot cm^{-1}$ in ethyl ether.

Photosensitizing activities of chlorophyll derivatives in rats

The photosensitizing activities of purified chlorophyll derivatives by single administration and 8 hr daily irradiation are summarized in Table IV. The 50% lethal doses for OH-Phd, Phd and Pyrophd were calculated to be approximately 31, 368 and 47 mg/kg of rats, respectively. Rats, when given the 100% lethal doses of pheophorbides (Phd's) and irradiated, soon became restless and then lethargic, and died in the period between 6 hr and 4 days after the onset of irradiation. Generally, externally apparent skin lesions had not developed at the time of death.

However, exploratory examinations after death revealed the following symptoms: 1, occurrence of serious lesions of villi in the small intestine; 2, destruction of lymphatic vessels; 3, falling off of epithelial cells; 4, swelling of intestinal tract by pools of much tissue fluid (presumably plasma effused from minute vessels); 5, slight congestion in parts of the liver, heart and lung; and 6, erythema beneath the skin of the back. Hematological tests showed marked increases of blood cells, hemoglobin and hematocrit in the blood.

The rats administered with over 100 mg of Phd/kg rats which survived showed erythema of the ears, dropsy of the face, neck and back and subsequent these lesions progressed to necrosis and depilation after a few days. On the other hand, the rats administered with OH-Phd and Pyrophd which survived did not show externally apparent lesions. Chl's *a* and *b*, pheophytin *a*, methyl and ethyl Phd, the mixture of pigments (P-6, P-7, P-8, P-9) located below the spot of OH-Phd (P-5) in the TLC in Fig. 1, and P-7 (*Rf* 0.21) were inactive in a single dosage of over 200 mg/kg.

Control rats kept in the dark after being administered with test pigments did not show any apparent symptoms in the external and internal organs.

The photosensitivity responses which occur-

TABLE IV. PHOTOSENSITIZING ACTIVITIES OF THE CHLOROPHYLL DERIVATIVES IN RATS IN A SINGLE ORAL DOSAGE

	Dose (mg/kg)	Photosensitivity response	
		No. of deaths / No. of rats irradiated	No. of rats with skin lesions* / No. of survivors
Chlorophyll *a*	500	0/3	0/3
Chlorophyll *b*	200	0/3	0/3
Pheophytin *a*	500	0/3	0/3
Methyl pheophorbide *a*	200	0/3	0/3
Ethyl pheophorbide *a*	400	0/3	0/3
Pheophorbide *a* (P-4)	700	10/10	—
	600	7/10	3/3
	500	6/10	4/4
	400	6/10	4/4
	300	5/10	5/5
	200	0/10	7/10
	100	0/10	3/10
	50	0/10	0/10
10-Hydroxy-pheophorbide *a* (P-5)	100	10/10	—
	75	6/10	0/4
	50	6/10	0/4
	25	5/10	0/5
	12.5	1/10	0/9
	6.25	0/10	0/10
(P-7)	200	0/3	0/3
Mixed pigments (P-6, 7, 8, 9)	200	0/3	0/3
Pyropheophorbide *a* (P-t)	100	10/10	—
	75	8/10	1/2
	50	6/10	0/4
	25	0/10	0/10

* The skin lesions refer only to those externally apparent in the rats.

red in rats by daily oral administration of Phd, OH-Phd and Pyrophd are summarized in Table V. Tests were performed for 90 days on the surviving rats. By daily administration with Phds above 0.78 mg of OH-Phd, 9.4 mg of Phd or 0.78 mg of Pyrophd/kg of rats, some photosensitivity responses including death were observed in rat, as in the case of a single dosage.

Rats administered daily with 0.78 mg of OH-Phd or 1.56 mg of Pyrophd which survived, grew normally and had no apparent skin lesions. However, histopathological examinations on the surviving rats at the end of the test revealed that there was appreciable change in the spleen of some rats, but not in the other internal organs. No abnormality was observed in rats administered daily for 90 days with 0.39 mg of OH-Phd or Pyrophd, or 4.7 mg of Phd.

DISCUSSION

Besides Phd, a new pigment causing intense photosensitivity in rats was isolated from alcohol-treated *Chlorella* cells and identified as OH-Phd. The photosensitizing activity of OH-Phd in rat was markedly higher than those of

TABLE V. PHOTOSENSITIVITY RESPONSES IN RATS ADMINISTERED PHEOPHORBIDE *a*, 10-HYDROXYPHEOPHORBIDE *a* AND PYROPHEOPHORBIDE *a* IN DAILY ORAL DOSAGE

Pigment	Dose (mg/kg rat/day)	Photosensitivity response		
		Deaths	Skin	Internal organs
Pheophorbide *a*	150	6/6*	+	(+)
	75	5/6	+	(+) 1/1**
	37.5	3/6	+	(+) 3/3
	18.8	4/6	−	(+) 0/2
	9.4	3/6	−	(+) 0/3
	4.7	0/6	−	− 0/6
10-Hydroxy-pheophorbide *a*	6.25	6/6	−	(+)
	3.13	3/6	−	(+) 2/3
	1.56	1/6	−	(+) 3/5
	0.78	1/6	−	(+) 2/5
	0.39	0/6	−	− 0/6
Pyropheophorbide *a*	25	6/6	−	(+)
	12.5	6/6	−	(+)
	6.25	3/6	−	(+) 1/3
	3.13	3/6	−	(+) 2/3
	1.56	2/6	−	(+) 2/4
	0.78	1/6	−	(+) 0/5
	0.39	0/6	−	− 0/6

+, externally apparent skin lesions; (+), histopathological changes beneath the skin or in some internal organs; −, no sign.

* Number of deaths/number of rats photoirradiated.

** Number of rats responding/number of survivors.

Phd and Pyrophd, well-known photosensitizing pigments having been reported to exist in process *Chlorella*,[2,13] lucerne leaf protein concentrate,[14] pickled greens[3,4,13,15] and abalones.[16]

There are a few previous studies on OH-derivatives of Chl. The chemical properties of methyl ester of OH-Phd, its visible and IR absorption spectra, HCl number and phase test were almost similar to those of 10-oxymethyl pheophorbide *a* reported by Holt[17] as a Mg-free derivative of one of the allomerization products of methyl Chlorophyllide *a*. However, 10-oxy-pheophorbide *a* prepared according to method of Holt differed from our OH-Phd in the ratio of the E_{667}/E_{409} (1.99), the value of *Rf* (0.21) in the TLC and the chemical shifts of the 7- and 8-protons in the NMR spectra. The analytical values of Holt's pigment were identical to those of P-7 shown in the present paper, which was presumed to be an optical isomer of OH-Phd and showed less photosensitizing.

Pennington *et al.*[18] have definitely established the structure of green substances produced by enzymatic oxidation of Chl's in plants (dandelion leaves, hollyhock leaves, barley seedling and cocklebur) as 10-hydroxychlorophylls *a* (OH-Chl) and *b*, but have made no mention of 10-hydroxychlorophyllide *a* (OH-Chld) and OH-Phd.

Although full details on the formation of OH-Phd in the *Chlorella* cells will be given in a subsequent paper, it may be summarized as follows: when a suspension of killed *Chlorella* cells maintaining an active enzyme system, such as freeze-dried, insufficiently heated (40 to 70°C) or nutrient deficient cells, was exposed to light and oxygen, the celular Chl was converted rapidly to OH-Chl, which was degraded rapidly to OH-Chld and OH-Phd in an aqueous organic solvent such as alcohol or

acetone. It was necessary for the formation of OH-derivatives of Chl that the cell suspension was shaken with oxygen for several hours. Under anaerobic conditions, OH-derivatives were not formed at all.

Pyrophd and phylloerythrine[19] were not detectable in alcohol-treated *Chlorella* cells. The principal active pigment of pickled greens (*Brassica juncea*) isolated by us was found to be Pyrophd. It has been reported[6,19] that these pigments are derived from Chl in the process of digestion in ruminants fed on grass, presumably due to decarbomethoxylation catalyzed by bacterial enzymes. In our case, the pigments were not detected in the feces and caecum contents in rats fed on *Chlorella* cells. The *Chlorella* cells used in the present study were bacteria free, but outdoor-cultured cells are naturally accompanied by bacterial contamination. Reassessment for the active pigments of such *Chlorella* cells may be required.

Chl's *a* and *b*, pheophytin *a*, and methyl- and ethyl-Phd's were inactive, but chlorophyllide *a* (Chld) and OH-Chld isolated from alcohol-treated *Chlorella* cells were active (data not shown) under our experimental conditions. Chlorophyllides and Phd's were not formed by treating *Chlorella* cells with aqueous organic solvent in the case where the cellular enzyme system had been inactivated by heating. Chl and pheophytin *a* were not degraded to Phd by treatment with 17% HCl. The findings presented in this paper suggest that propionic acid residue at C-7, apparent from the chlorophyllase activity, and the groups situated at C-10 in Phd's would participate essentially in the photochemical reactions of Phd's and also in the absorption of them by animal tissues.

The molar absorption coefficients at the red max. of three Phd's and their methyl esters were measured in ethyl ether. The values for methyl Phd, methyl OH-Phd and methyl Pyrophd were 56,200, 57,600 and 52,000 $M^{-1} \cdot cm^{-1}$, respectively. The value for methyl Phd was close to the value reported by Pennington *et al.*[6] and by Holt and Jacobs.[10] Holt and Jacobs found that their values for the molar absorption coefficient of ethyl Phd's were almost completely similar to those reported for the pheophytins by Zscheile and Comar.[9] Therefore, they concluded that the phytyl group does not influence the molar absorption coefficient. Our values for Phd's are clearly less than those of the respective methyl esters. The values 41,600 $M^{-1} \cdot cm^{-1}$ of Phd in ethyl ether reported by Brown[20] and 44,900 $M^{-1} \cdot cm^{-1}$ by Wickliff and Aronoff[21] are also less than the value having expected by them and our value in the present paper. We are not certain why the values for Phd's are low, but it seems that a cause lies in the lower solubility of Phd's in preparation of the ether solution, which was done as described in the measurement of absorption spectra.

It is obvious that the photosensitivity in rats by Phd's was caused by their photodynamic action since the diseases did not occur in the dark, but did by irradiation with visible light. Photodynamic action[22] is basically a photo-initiated oxidation involving energy transfer from the sensitizer excited by visible light to oxygen to form excited singlet oxygen as a reactive and short-lived intermediate, which consequently causes inactivation of biomolecules and biological systems.

Isobe *et al.*[4] have reported that lethal photosensitivity in rats caused by intraperitoneal administration of Phd was induced from hemolysis, which was produced by peroxidation of lipid in the red blood cell membranes. Our results show that the lethal photosensitivity in rats caused by oral administration of Phd's was induced from lesions primarily occurring on the villi in the small intestine. These serious lesions markedly increased the vascular permeability in the tissue, then effused much plasma from minute vessels to the intestinal tract, resulting in an acute circulatory insufficiency, then death of the rats. The direct action of light on the internal organs in the rat was also supported by the experimental results that an effective light reached the internal organs through the skin in rat. This finding suggests that the photosensitization of animals is affected

not only by the activity of the pigments, but also by the color or size of the animals which has influence of the penetration of light. It may be assumed that primary photosensitivity signs in large animals such as human do not occur in the internal organs, but in the skin because of the fact that an effective light hardly reaches their internal organs. Therefore, the lethal mechanisms and the lethal doses in rats of Phd's do not apply in the case of large animals. More detailed pathological studies on the photosensitivity caused by Phd's will be described in a subsequent paper.

Acknowledgments. The authors are grateful to Professor Tsutomu Sasa of Miyazaki Medical College for his valuable advice concerning chlorophyll derivatives, to Dr. Kimio Higashiyama of the Hoshi College of Pharmacy for his NMR measurements and to Mr. Kazumi Uchida of the our Institute for his histopathological examinations.

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