

antimutagenicity towards MNNG. In particular, the structural differences relating to the presence or absence of the phytol or methyl groups, which influence hydrophobic properties (pyropheophytin *a* > pheophytin *a* > pheophorbide *a*), did not affect their antimutagenic efficacy against 3-MC. Such results suggest that the antimutagenic properties of these compounds towards the indirect-acting mutagen, 3-MC, may involve the porphyrin nucleus and is, for the most part, independent of the configuration of the side chains. Tachino et al. [22] have suggested that a possible mechanism for the antimutagenic activity of chlorophyll derivatives involves complex formation with the mutagen through strong  $\pi$ - $\pi$ -interactions between their planar unsaturated cyclic rings. This may be the case with 3-MC. Difference spectral analysis in the visible region, capable of detecting perturbations of the electron distribution of the porphyrin nucleus in the presence of a large molar excess of 3-MC, indicates that interaction between pheophytin *a* or pheophorbide *a* and mutagen does occur. This is most evident by changes of absorbencies in the Soret and Q bands (Soret/Q ratio). The impact of complexation on the principal absorption peaks of the chlorophyll derivatives allows for a sensitive detection of interaction. This type of analysis possesses an internal control of experimental variables since it is based upon a ratio of absorbencies at two different wavelengths of the same sample. The Soret/Q ratio may be especially valuable in detecting the direct interaction of chlorophyll derivatives and mutagens when the complexation is of non-covalent nature and relatively weak, as found for some mutagens [24].

It should be noted that inhibition of the mutagenicity of 3-MC by a complex formation does not exclude that pheophytin *a*, pyropheophytin *a*, and pheophorbide *a* may also inactivate the enzymatic transformation of this mutagen. It is possible that two different mechanisms may be involved simultaneously in desmutagenic activity of chlorophyll derivatives against indirect-acting mutagens. Thus, the results from the study by Yun et al. [25] indicate that CHL may inhibit the mutagenicity of 2-amino-3-methylimidazo[4,5-f]quinoline through inactivation of cytochrome P450 in addition to complexation with this mutagen.

In contrast to the results seen with 3-MC, the mutagenicity of MNNG is inhibited differently by pheophytin *a*, pyropheophytin *a*, or pheophorbide *a*, suggesting the important role of the phytol chain in such an activity. The unchanged Soret/Q ratios imply that the complexation of MNNG with any of the chlorophyll derivatives does not occur. This is consistent with the fact that MNNG lacks an extended planar,  $\pi$ -electron cloud required for interaction with the  $\pi$ -system of porphyrin type compounds. Romert et al. [26] suggested that a possible mechanism of inactivation involves the decomposition of MNNG mediated by chlorophyll derivatives similar to that for thiols and amines. In particular, CHL may facilitate the formation of highly mutagenic intermediates derived from MNNG that are immediately inactivated as a result of the reaction with water outside the cell.

The potential of chlorophyll derivatives as antimutagens raises questions about their affect on normal and pathological tissue. Porphyrin-related compounds, and chlorophyll derivatives in particular, have a high affinity for tumor tissue as compared to normal tissue [27]. There is also evidence that individual chlorophyll derivatives, as well as commercial preparations derived from chlorophyll, have cytostatic and cytotoxic activities against tumor cells. Tissue culture studies showed that pheophytin is cytostatic against hepatoma cells [28] and along with chlorin *e<sub>6</sub>* and CHL, has a cytotoxic effect on myeloma cells [10,11]. The chlorophyll derivatives

studied here are cytostatic and at higher concentrations are cytotoxic to murine myeloma cells. One factor, however, that plays an important role in such activities is the ability of the porphyrins to become cell associated. The approaches employed in our study utilizing cell bound fluorescence or chemical extraction of chlorophyll derivatives not associated with cells exhibit similar patterns for cellular uptake of pheophytin *a* and pheophorbide *a*. Both procedures demonstrate pheophorbide *a* accumulates to a higher molar concentration than pheophytin *a* when myeloma cells are exposed to equimolar doses of each compound. Although the fluorescence procedure indicates much greater differences than the chemical extraction method in the ability of these compounds to become cell associated, the same trends are observed. Aggregation and/or formation of metal complexes may occur within the cell, thereby affecting the fluorescence yield of the cell bound chlorophyll derivatives [29,30]. In turn, these factors may impact pheophytin *a* and pheophorbide *a* differently. Accordingly, the chemical extraction approach is taken as more reliable in quantifying difference between these compounds. Pheophorbide *a* exhibits a significantly higher cytostatic/cytotoxic effect than pheophytin *a* when calculated on the amount added to the incubation medium. However, when such values are adjusted for the amount of cell associated chlorophyll derivatives, pheophytin *a* is more cytostatic and cytotoxic for the myeloma cells than pheophorbide *a* (3.2 and 5.0 times more active for multiplicity and viability, respectively). The greater cellular uptake of pheophorbide *a* in myeloma cells as compared to murine splenocytes found in this study is in agreement with the data employing MGH U1 cells, a line established from a human transitional cell carcinoma of the bladder, and IMR-90, a non-neoplastic line established from human fibroblasts [31].

Given the importance of antimutagenic/anticarcinogenic activities coupled with selective accumulation in tumor tissue and cytostatic/cytotoxic effects against tumor cells, chlorophyll derivatives might be a significant tool in future management of cancer.

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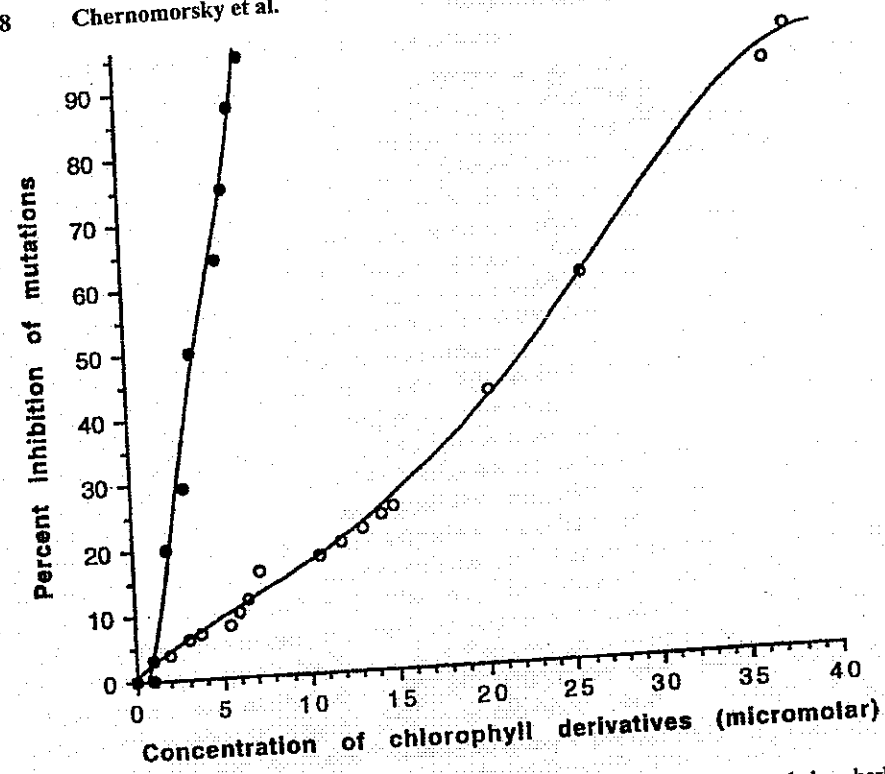


Fig. 3. Antimutagenic activity of pheophytin *a* and pyropheophytin *a* (closed circle) and pheophorbide *a* (open circle) against MNNG.

*a* to cause similar effects. The impact of both chlorophyll derivatives on cell viability, however, is identical (0.66 and 1.30 mM for ED<sub>50</sub> and ED<sub>95</sub>, respectively).

In order to further understand the possible mechanism(s) involved in the cytotoxic/cytostatic activities of these chlorophyll derivatives, the distribution of each compound between the cells and medium was determined. Table II demonstrates that pheophorbide *a* which lacks the phytol ester group and possesses a free carboxylate moiety as compared to pheophytin *a* exhibits a 5-fold greater cellular uptake than the latter. The cell/medium distribution of pheophorbide *a* in non-tumor cells, murine splenocytes, was found to be significantly less (by 25 times) than that in myeloma

TABLE I. Spectral Characteristics of Chlorophyll Derivatives and Their Mixture With Mutagens

Compounds	Soret band <sup>a</sup>	Q band <sup>a</sup>	Soret/Q <sup>b</sup>
Pheophytin <i>a</i> alone	413	666	2.9
Pheophytin <i>a</i> + 3-MC	413	666	2.3
Pheophytin <i>a</i> + MNNG	413	666	2.9
Pheophorbide <i>a</i> alone	413	666	3.2
Pheophorbide <i>a</i> + 3-MC	413	666	1.4
Pheophorbide <i>a</i> + MNNG	413	666	3.2

<sup>a</sup>Wavelength of maximum absorption, nm.

<sup>b</sup>Ratio of the absorbances at the maxima of the Soret and Q bands.

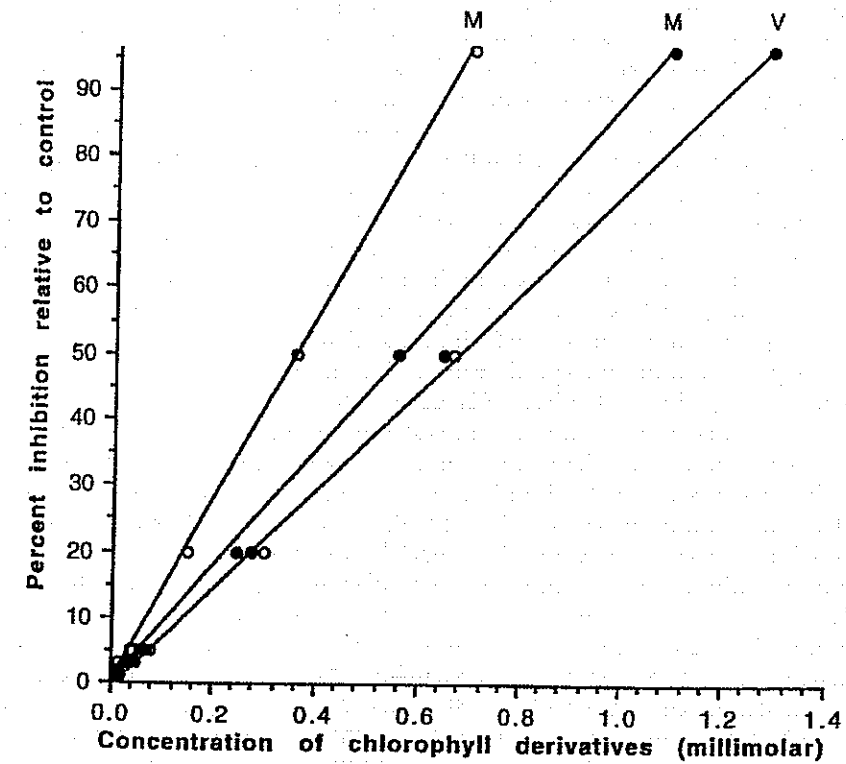


Fig. 4. Effect of pheophytin *a* (closed circle) and pheophorbide *a* (open circle) on the growth (M-multiplicity, V-viability) of murine myeloma cells.

cells. These observations are supported by the determination of the cell bound chlorophyll derivatives detected by cellular fluorescence. The fluorescence method, however, indicated a much greater difference in uptake values of the chlorophyll derivatives than those from the spectrophotometric measurements.

## DISCUSSION

Clearly, pheophytin *a*, pyropheophytin *a*, and pheophorbide *a* each exhibit antimutagenicity towards 3-MC or MNNG. Interestingly, the three chlorophyll derivatives demonstrate identical activities against 3-MC but differ in regard to

TABLE II. Uptake of Chlorophyll Derivatives by Tumor and Non-Tumor Cells

Compounds	Cells	Distribution <sup>a</sup>	Relative fluorescence/cell
Pheophytin <i>a</i>	Myeloma cells	0.10	2
Pheophorbide <i>a</i>	Myeloma cells	0.50	264
Pheophorbide <i>a</i>	Murine splenocytes	0.02	4

<sup>a</sup>Ratio of the concentrations of chlorophyll derivatives in cells and medium.

### Cell Cultures, Cytotoxicity, and Uptake Studies

Tumor cells (mouse myeloma line P3X63Ag8), an azaguanine resistant P3 clone derived from MOPC-21, were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 50 IU/ml penicillin, and 50 µg/ml streptomycin (Gibco Laboratories, Grand Island, NY), at 37°C in an environment of 10% CO<sub>2</sub>/90% air. Cells suspended in fresh culture medium to give 3 × 10<sup>5</sup> cells/ml were incubated with pheophytin *a* and pheophorbide *a* as described previously [10]. Splenocytes, obtained from the C3H mice, were suspended in Hanks' balanced salts solution, centrifuged, and resuspended in 1 ml of distilled water for 15 s to lyse erythrocytes [18]. After dilution with Hanks' balanced salts solution, cells were pelleted by centrifugation, washed, and resuspended to 10<sup>6</sup> cells/ml of the same solution. Ethanolic solutions of chlorophyll derivatives were mixed with the cells. The concentration of ethanol did not exceed 10% of the total volume and was found to be non-toxic to cells at this concentration.

After an incubation period of 48 h with the chlorophyll derivatives, viability of myeloma cells was determined by trypan blue exclusion. Multiplicity of myeloma cells was assessed as a ratio of the total number of cells after exposure to pheophytin *a* or pheophorbide *a* to that in control cultures [10]. For cellular uptake studies, cells were incubated for 24 h with each chlorophyll derivative (230 µM). Following centrifugation (500g) for 30 min, the supernate was reserved and cells were washed three times with fresh phosphate-buffered saline. The combined medium and cell washings were extracted with a mixture of acetone and diethyl ether (1.4:1). The organic layer was removed, washed with water, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and analyzed spectrophotometrically for the respective chlorophyll derivatives. The amount of compound recovered was compared with that added to the cells prior to incubation.

In addition, the fluorescence of cells incubated with the chlorophyll derivatives was detected by using a Leitz Diavert microscope equipped with epi-illumination and an MPV compact photometer (excitation 350–460 nm, emission 515 nm).

## RESULTS

### Antimutagenic Activity

Figure 2 demonstrates that all of chlorophyll derivatives tested are antimutagenic against 3-MC. They exhibit similar dose-response patterns, reaching a level of ED<sub>50</sub> and ED<sub>95</sub> (effective doses required to afford 50 or 95% decrease in the number of bacterial cells) at the 0.13 and 0.89 µM, respectively. Quite different responses are evident with MNNG. As seen in Fig. 3, the 50 and 95% inhibition of His<sup>+</sup> revertants requires much higher concentrations for pheophorbide *a* (24.0 and 38.5 µM, respectively) compared to pheophytin *a* and pyropheophytin *a* (3.9 and 6.9 µM, respectively).

The antimutagenic effect of pheophorbide *a* or pheophytin *a*, as reported here, is supported in separate experiments by others, though with different indirect-acting mutagens. The activity of these chlorophyll derivatives, however, was not determined at identical concentrations of the mutagens and accordingly the dose-response curves are difficult to compare [19,20].

### Spectral Changes of Chlorophyll Derivative/Mutagen

Evidence suggests that chlorophyll and CHL are involved in molecular complexation with a number of conjugated polycyclic aromatic mutagens [21–23], thereby

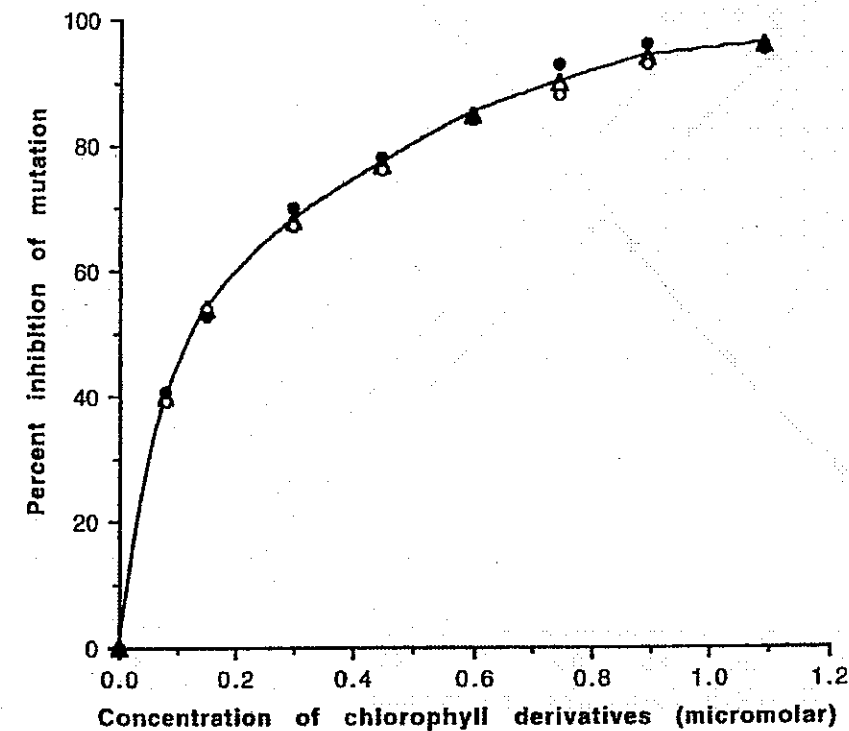


Fig. 2. Antimutagenic activity of pheophytin *a* (closed circle), pyropheophytin *a* (open triangle) and pheophorbide *a* (open circle) against 3-MC.

reducing or totally eliminating their bioavailability. In these studies, difference spectra of chlorophyll or CHL mixed with mutagen were obtained. The spectral changes were indicative of the complex formation. We compared the visible absorption spectra of MNNG or 3-MC mixed with pheophytin *a* or pheophorbide *a* against that of the appropriate mutagen and chlorophyll derivative alone (Table I). The results revealed that the position of the Soret and Q bands for pheophytin *a* or pheophorbide *a* remained unchanged in the presence of either MNNG or 3-MC. However, the ratios of the absorption for these bands are different depending on the mutagen used. The ratios for chlorophyll derivatives mixed with MNNG are the same as for those compounds alone. In contrast, 3-MC added to pheophytin *a* or pheophorbide *a* decreases the ratios of the Soret/Q bands by 20% and 57%, respectively. This pattern also is observed in studies with CHL and benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide [22].

### Cytotoxic Effect and Cellular Uptake

The dose-effect profiles of chlorophyll derivatives with respect to growth properties of myeloma cells are shown in Figure 4. Pheophorbide *a* is approximately 55–57% more effective as an inhibitor of cell multiplicity than pheophytin *a*. It can be seen that pheophorbide *a* requires concentrations of only 0.36 and 0.70 mM for ED<sub>50</sub> and ED<sub>95</sub>, respectively. Concentrations of 0.56 and 1.10 mM are needed for pheophytin

## INTRODUCTION

Along with other dietary constituents of vegetables tested for their effect on chemically induced mutagenesis/carcinogenesis, much attention in recent years has been given to chlorophyll and its water-soluble commercial version, chlorophyllin copper complex (CHL) [1-4]. A close positive relationship was established between the chlorophyll content of various vegetable extracts and their ability to inhibit mutations in the Ames *Salmonella* system [5-7]. Extracts from parsley and lettuce, which have high levels of chlorophyll, were more effective in modulating mutagenicity caused by 3-methylcholanthrene (3-MC) and benzo[*a*]pyrene (BP) compared to other common vegetables in the diet of the Western population [6]. Such a correlation with chlorophyll content was also found among the vegetables favored by the Oriental (Korean) population. Extracts of mustard leaves and Korean radish leaves exhibited the strongest antimutagenic activity against *N'*-nitro-*N'*-nitrosoguanidine (MNNG) [7]. Chlorophyll was also tested individually towards cigarette-smoke condensate and BP [8]; and 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole (Trp-P-2) and 3-hydroxiamino-1-methyl-5H-pyrido[4,3-*b*]indole [1] and 4-nitro-*o*-phenylenediamine [9]. For all mutagens used, chlorophyll demonstrated an inhibitory effect, though various conditions were required to fully reveal its potency.

The antimutagenic effect of chlorophyll and its derivatives has been well documented. Recently, limited data appeared on the tumoricidal activity of these compounds in a tissue culture model. For example, CHL was shown to exhibit considerable cytotoxicity against myeloma cells [10,11].

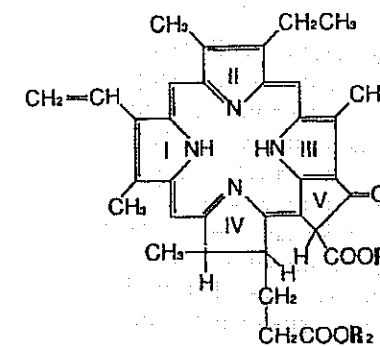
CHL, the most common among chlorophyll derivatives used in cancer related studies, is a semi-synthetic preparation. Its parent compound, chlorophyll, however, is a naturally occurring porphyrin. It is noteworthy that chlorophyll is known to be converted into pheophytin, pyropheophytin, and pheophorbide (Fig. 1) in stored plant tissues, processed vegetable food, and following ingestion by humans [12-14]. In this report, we present data on the antimutagenic and tumoricidal potencies of these compounds that may contribute to the practical application of chlorophyll and its derivatives as dietary supplements in cancer prevention.

## MATERIALS AND METHODS

## Chemicals

Chlorophyll *a*, isolated from *Anacystis nidulans* algae, MNNG, and 3-MC were purchased from Sigma Chemical Co. (St. Louis, MO). Cell culture media, other chemicals and reagents for mutagenicity, and cytotoxicity assays were obtained from Fisher Scientific (Pittsburgh, PA). Chlorophyll *a* was the source for the preparation of various chlorophyll derivatives.

Pheophytin *a* was produced from a diethyl ether solution of the chlorophyll *a* by acidification with 13% aqueous HCL. HCL was then removed by washing with distilled water. The diethyl ether solution was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated in vacuo [15]. Pyropheophytin *a* was prepared by heating a pyridine solution of pheophytin *a* in a sealed tube at 100°C. After 48 h, the pyridine was removed by evaporation in vacuo and the pyropheophytin *a* was obtained as a residue [16]. Pheophorbide *a* was prepared by treating a diethyl ether solution of the chlorophyll *a* with 30% (w/w) aqueous HCL in the dark for 1 h at room temperature. The mix-



**Pheophytin *a* :**  
**R<sub>1</sub>-CH<sub>3</sub>; R<sub>2</sub>-C<sub>20</sub>H<sub>39</sub>**  
**Pyropheophytin *a* :**  
**R<sub>1</sub>-H; R<sub>2</sub>-C<sub>20</sub>H<sub>39</sub>**  
**Pheophorbide *a* :**  
**R<sub>1</sub>-CH<sub>3</sub>; R<sub>2</sub>-H**

Fig. 1. Structures of chlorophyll derivatives.

ture was diluted with distilled water and pheophorbide *a* in the ether phase was washed with distilled water to remove HCL. The ether solution was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and the solvent was removed by evaporation in vacuo [17].

## Mutagenicity Studies

*Salmonella typhimurium* strain TA100 was employed as a test system following the procedure described previously [10]. All chlorophyll derivatives were initially dissolved in dimethylsulfoxide and added, at concentrations as noted, to the media containing the bacterial cells and the appropriate mutagen. The amount of the indirect-acting mutagen, 3-MC (50 µg/plate), to cause 1,320 His<sup>+</sup> revertants, or the direct-acting mutagen, MNNG (5 µg/plate), to cause 478 His<sup>+</sup> revertants, were employed as standard conditions. The counts were corrected for spontaneous background revertants.

## Chlorophyll Derivative/Mutagen Interaction

The absorption spectrum of pheophytin *a* and pheophorbide *a* alone or mixed with mutagens (at chlorophyll derivative/mutagen molar ratios of 1:10) in dimethylsulfoxide were obtained in a range between 350-700 nm by using a computer controlled Perkin-Elmer Lambda 3B UV-VIS spectrophotometer.

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## Effect of Dietary Chlorophyll Derivatives on Mutagenesis and Tumor Cell Growth

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Much attention in recent years has been given to the antigenotoxicity of chlorophyll. Chlorophyll, however, is known to be converted into pheophytin, pyropheophytin, and pheophorbide in processed vegetable food and following ingestion by humans. Studies were conducted on the antimutagenic and tumoricidal potencies of these compounds. All the chlorophyll derivatives tested exhibit identical antimutagenic effect towards 3-methylcholanthrene (3-MC), suggesting that the porphyrin nucleus may complex directly with the mutagen. It does not exclude, however, another mechanism of activity involving inactivation of the enzymatic transformation of 3-MC. In contrast, the action of *N*-nitro-*N*'-nitrosoguanidine (MNNG) depends upon structural differences between the chlorophyll derivatives. It is significantly lower when the phytol-containing pheophytin and pyropheophytin are tested as to that of the phytol-lacking pheophorbide. The higher concentrations of the chlorophyll derivatives were required to reduce the mutagenicity of MNNG than needed for 3-MC. The cytotoxicity of chlorophyll derivatives against tumor cells also was evaluated. The cellular uptake and inhibition of myeloma cell multiplicity were found to be greater for pheophorbide than for pheophytin. Calculated on the amount of cell associated chlorophyll derivative, however, pheophytin was more cytostatic/cytotoxic than pheophorbide. The results presented in this report indicate that food sources that yield chlorophyll derivatives may play a significant role in cancer prevention. *Teratogenesis Carcinog. Mutagen.* 19:313-322, 1999. © 1999 Wiley-Liss, Inc.

**Key words:** chlorophyll derivatives; pheophytin; pyropheophytin; pheophorbide; antimutagens; cytotoxicity

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