Natural Chlorophyll but Not Chlorophyllin Prevents Heme-Induced Cytotoxic and Hyperproliferative Effects in Rat Colon¹,²

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ABSTRACT Diets high in red meat and low in green vegetables are associated with an increased risk of colon cancer. In rats, dietary heme, mimicking red meat, increases colonic cytotoxicity and proliferation of the colonocytes, whereas addition of chlorophyll from green vegetables inhibits these heme-induced effects. Chlorophyllin is a water-soluble hydrolysis product of chlorophyll that inhibits the toxicity of many planar aromatic compounds. The present study investigated whether chlorophyllins could inhibit the heme-induced luminal cytotoxicity and colonic hyperproliferation as natural chlorophyll does. Rats were fed a purified control diet, the control diet supplemented with heme, or a heme diet with 1.2 mmol/kg diet of chlorophyllin, copper chlorophyllin, or natural chlorophyll for 14 d (n = 8/group). The cytotoxicity of fecal water was determined with an erythrocyte bioassay and colonic epithelial cell proliferation was quantified in vivo by [methyl-³H]thymidine incorporation into newly synthesized DNA. Exfoliation of colonocytes was measured as the amount of rat DNA in feces using quantitative PCR analysis. Heme caused a >50-fold increase in the cytotoxicity of the fecal water, a nearly 100% increase in proliferation, and almost total inhibition of exfoliation of the colonocytes. Furthermore, the addition of heme increased TBARS in fecal water. Chlorophyll, but not the chlorophyllins, completely prevented these heme-induced effects. In conclusion, inhibition of the heme-induced colonic cytotoxicity and epithelial cell turnover is specific for natural chlorophyll and cannot be mimicked by water-soluble chlorophyllins. J. Nutr. 135: 1995–2000, 2005.

KEY WORDS: • diet • red meat • green vegetables • prevention • colon cancer

Colon cancer was responsible for >0.5 million deaths worldwide and was the second leading cause of cancer death in Western countries in 2000 (1,2). Risk factors for colon cancer include a positive family history or environmental factors, with diet as a major modulator. In particular, diets high in red and processed meat, in contrast to white meat, are associated with increased colon cancer risk (3–5). However, people who consume a substantial amount of green vegetables have a reduced risk of colon cancer (6).

The mechanisms explaining the dietary modulation of the risk of colon cancer by intake of red meat and vegetables are still under debate. One mechanism was deduced from nutritional studies with rats. Sesink et al. (7) showed, in rats, that dietary heme (Fig. 1A), the iron-porphyrin pigment of red meat, increased cytotoxicity of the fecal stream. This resulted in increased exposure of the colonocytes to luminal irritants (7). Consequently, colonocyte proliferation increased, which is considered an important risk factor in the development of colon cancer (8–11). In line with these results, Pierre et al. (12,13) showed that dietary heme or meat supplemented to the diet promoted luminal cytotoxicity and increased the number and size of aberrant crypt foci in rat colon. Abnormal crypt foci are preneoplastic lesions that correlate with tumor incidence in most studies (14).

Remarkably, supplementation of this heme diet with freeze-dried spinach or an equimolar amount of natural chlorophyll inhibited the heme-induced cytotoxic and hyperproliferative effects (15). Chlorophyll (Fig. 1B) is the ubiquitous pigment in green leafy vegetables; like heme, it also has a planar porphyrin backbone. Chlorophyll is different from heme mainly by having the nonreactive magnesium instead of the highly reactive transition metal iron in the center of the porphyrin. In addition, chlorophyll has an esterified phytol tail instead of a propionic side chain. We speculated that chlorophyll “sandwiches” heme to form hydrophobic heme-chlorophyll complexes; as a result, it blocks radical-mediated heme metabolism and the heme-induced changes in epithelial cell turnover (15). In this study, we wanted to investigate whether this protective effect of natural chlorophyll is due to its porphyrin ring or requires the intact phytol-conjugated molecule. There-
fore, we compared the possible protective effect of chlorophyllins with that of natural chlorophyll.

Chlorophyllins (Fig. 1C) are molecular analogs of chlorophyll studied for cancer prevention in vitro and in vivo because they might mimic the effects of chlorophyll (16–19). Chlorophyllins are food-grade molecules derived from chlorophyll. They are hydrophilic, due to hydrolysis of the phytol tail, and the magnesium in the center of the porphyrin ring is removed or replaced by another metal. In contrast to the limited in vivo studies with natural chlorophyll (20,21), chlorophyllins have received much more attention. Several studies indicate that chlorophyllins may have anticarcinogenic effects because their porphyrin macrocycle can either scavenge free radicals or form a complex with planar aromatic carcinogens and thus reduce their bioactivity (17,22,23).

Based on these data from the literature and because heme is also a planar aromatic compound, we suggest that the chlorophyllins block heme by complex formation. Blocking of heme by chlorophyllins might inhibit its metabolism in the gut lumen as chlorophyll does (15). Consequently, chlorophyllin might also prevent the heme-induced luminal cytotoxicity and increased colonic epithelial cell turnover. We tested this in rats, supplementing their diets with heme, heme plus chlorophyllins, or heme plus chlorophyll.

MATERIALS AND METHODS

Animals and diets. The experimental protocol was approved by the animal welfare committee of Wageningen University and Research Center. Outbred male SPF Wistar rats (~8 wk old; WU, Harlan) were housed individually in metabolic cages in a room with controlled temperature (~20°C), relative humidity (50–60%), and a 12-h light:dark cycle (lights on 0600–1800 h). Metabolic cages with wire-mesh bottoms made it possible to collect feces without urinary contamination. Rats were acclimated to the housing conditions for 5 d before the start of the experiment.

The body weight of the rats at the start of the experiment was 275 ± 1 g (mean ± SEM). For 2 wk, 5 groups of 8 rats were fed purified diets. The composition of the diets is given in Table 1. The heme-fed rats consumed a purified control diet supplemented with 0.5 mmol heme/kg diet (Sigma-Aldrich Chemie); 3 additional heme groups consumed the purified control diet supplemented with 0.5 mmol heme/kg diet (Sigma-Aldrich Chemie); 3 additional heme groups

TABLE 1

Composition of the experimental diets

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control</th>
<th>Heme</th>
<th>Heme plus Na-chlorophyllin</th>
<th>Heme plus Cu-chlorophyllin</th>
<th>Heme plus chlorophyll</th>
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<tbody>
<tr>
<td></td>
<td>g/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid casein</td>
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<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
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<tr>
<td>Dextrose</td>
<td>532</td>
<td>532</td>
<td>531</td>
<td>531</td>
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<tr>
<td>Palm fat</td>
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<td>160</td>
<td>160</td>
<td>160</td>
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<td>Corn oil</td>
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<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Heme</td>
<td>—</td>
<td>0.326</td>
<td>0.326</td>
<td>0.326</td>
<td>0.326</td>
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<tr>
<td>Na-chlorophyllin</td>
<td>—</td>
<td>—</td>
<td>0.80</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cu-chlorophyllin</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.87</td>
<td>—</td>
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<tr>
<td>Chlorophyll</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CaHPO₄·2H₂O¹</td>
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<td>3.44</td>
<td>3.44</td>
<td>3.44</td>
<td>3.44</td>
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<tr>
<td>MgCl₂·6H₂O²</td>
<td>—</td>
<td>—</td>
<td>0.25</td>
<td>—</td>
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<tr>
<td>Mineral mix³</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
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<tr>
<td>Vitamin mix³</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
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</table>

¹ Calcium was added as calcium phosphate (CaHPO₄·2H₂O, Fluka Chemie Buchs, Switzerland).
² The heme plus Na-chlorophyllin diet was also supplemented with 1.2 mmol/kg magnesium chloride to adjust for the magnesium content of chlorophyll.
³ The composition of the vitamin and mineral mixtures is according to the recommendation of the AIN (38), except that calcium was omitted. In addition, tri-potassium citrate was added instead of KH₂PO₄, and choline was added as choline chloride.
were supplemented with 1.2 mmol/kg Na-chlorophyllin, sodium copper chlorophyllin (Cu-chlorophyllin), (Haining FengMing Chlorophyll), or 1.2 mmol/kg chlorophyll (Japan Chlorophyll).

Food was administered to the rats just before dark to prevent possible degradation of the supplements. Food and demineralized drinking water were consumed ad libitum. Food intake and body weights were recorded every 2–4 d. Feces were collected quantitatively on d 11–14 of the experiment and frozen at −20°C. In vivo colonic epithelial cell proliferation. After 14 d of experimental feeding, colon epithelial cell proliferation was quantified in vivo by measuring DNA replication, using [methyl-3H]thymidine incorporation into DNA. Rats that had not been food deprived were injected i.p. with [methyl-3H]thymidine (specific activity 925 GBq/mmol, dose 3.7 MBq/kg body weight, Amersham International) in 154 mmol/L NaCl. After 3 h, rats were killed by CO2 inhalation. The colon was excised and opened longitudinally. Colonic contents were removed, and the mucosa was scraped, homogenized in buffer, and analyzed as described previously (15).

Quantification of epithelial DNA in feces. We quantified host DNA in feces as a marker for epithelial exfoliation, as described earlier (15,24). Briefly, fecal host DNA was extracted from freeze-dried feces. The DNA in all isolates was of good purity (A260/A280 ≈ 1.8). It was stored at 4°C, or −20°C for longer storage. The standard DNA used for quantification was isolated from rat spleen.

Quantification was based on real-time PCR, performed with rat-specific probe and primers targeted to the β-globin gene sequence (24).

Cytotoxicity of fecal water. Fecal water was prepared by reconstituting a small amount of freeze-dried feces with double-distilled water to obtain a physiologic osmolarity of 300 mOsmol/L, as described earlier (7). After preparation, the fecal waters were stored at −20°C until further analysis. Cytotoxicity of fecal water was quantified by potassium release of human erythrocytes after incubation with fecal water as described previously (7) and validated earlier with human colon carcinoma-derived Caco-2 cells (25). The potassium content of the erythrocytes was measured with an Inductive Coupled Plasma Absorption Emission Spectrophotometer (ICP-AES, Varian) and the cytotoxicity of fecal water was calculated and expressed as a percentage of maximal lysis.

Determination of heme in feces. The total amount of heme excreted in the feces was determined by a modified protocol of the HemoQuant assay (26). To quantify heme in feces, an acidified chloroform-methanol extract (27) was obtained from 30 mg of freeze-dried feces (final HCl concentration 1 mol/L). The chloroform phase was dried under nitrogen and dissolved in 0.45 mL of 250 mmol/L KOH. Subsequently, 0.45 mL of double-distilled water, 3.75 mL of 2-propanol, and 0.75 mL of 1.15 mol/L HCl were added to the samples, which were assayed for their heme content as described previously (26).

TBARS assay of fecal water. To determine lipid peroxidative processes in the lumen, TBARS in fecal water were quantified (7). The TBARS assay evaluates lipid peroxidation by quantifying the concentration of malondialdehyde (MDA) in fecal water (28).

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TBARS assay of fecal water. To determine lipid peroxidative processes in the lumen, TBARS in fecal water were quantified (7). The TBARS assay evaluates lipid peroxidation by quantifying the concentration of malondialdehyde (MDA) in fecal water (28). Briefly, fecal water was diluted 5-fold with double-distilled water. Subsequently, 100 μL of this solution was mixed with 100 μL of 8.1% SDS and 1 mL solution of 0.11 mol/L 2,6-di-tert-butyl-p-cresol, 0.5% TBA in 10% acetic acid (pH = 3.5). For background correction, TBA was omitted from the assay. TBARS were extracted, after heating for 60 min at 95°C, with 1.2 mL of n-butanol. The absorbance of this extract was measured at 532 nm (Spectramax plus, Bucher Biotec AG). The amount of TBARS was calculated as MDA equivalents using 1,1,3,3-tetramethoxypropane as standard.

Determination of distribution coefficients. A method similar to that described by Kepczynski et al. (29) was used to determine the octanol/water distribution coefficient of heme, Na-chlorophyllin, Cu-chlorophyllin, and chlorophyll. The supplements were dissolved in 2 mL of octanol or PBS at pH 7.3 (final concentrations < 4 μmol/L) and mixed with a vortex with an equal volume of PBS or octanol, respectively. After 20 min shaking and 10 min centrifugation at 3000 × g, concentrations in both phases were determined using a spectrophotometer (Lambda 2, Perkin Elmer). The distribution coefficients of the supplements were defined by their concentration in octanol divided by their concentration in PBS.

**RESULTS**

Addition of heme, chlorophyllins, or chlorophyll to the diets did not affect food intake (18.5 ± 0.5 g dry weight/d). Furthermore, the growth rate did not differ (4.1 ± 0.3 g/d) among the treatment groups.

We measured proliferation of the colonocytes in vivo to examine whether heme, chlorophyllins, or chlorophyll in the diet changed the response of the epithelial cells. The hemesupplemented group had an almost 100% increase in proliferation compared with the nonheme control group (Fig. 2A).

Adding chlorophyllins to the heme diet did not inhibit the heme-induced hyperproliferation of colonic cells. In contrast, supplementing the heme diet with natural chlorophyll inhibited the heme-induced proliferation to a level similar to control values.

We quantified the level of host DNA in feces to study...
diet-induced differences in proliferation coincided with changes in exfoliation of the colonic epithelial cells (15). Compared with the control diet, dietary heme markedly reduced the amount of host DNA in the feces (Fig. 2B). Cu-chlorophyllin added to the heme diet did not inhibit this heme-induced effect; addition of Na-chlorophyllin to the heme diet slightly increased the level of host DNA in feces compared with the effect of heme. However, adding chlorophyllin to the heme diet completely prevented the heme-induced decrease in fecal host DNA excretion.

We analyzed cytotoxicity of the fecal waters with an erythrocyte bioassay to determine whether the changes in colonic epithelial cell turnover resulted from differences in exposure to luminal irritants. Cytotoxicity of the fecal water from the heme group was 90%, which was >50-fold higher than in the control group (Fig. 3). The heme-induced cytotoxicity decreased significantly in the Na-chlorophyllin-supplemented heme group to 57%. In contrast, heme-induced cytotoxicity was blocked completely when chlorophyllin was added to the heme diet.

We showed earlier that the detrimental heme-induced effects are mediated by luminal conversion of ingested heme (15,30). Therefore, we studied whether the dietary treatments affected intestinal heme metabolism. The amount of daily heme intake and excretion in the feces (Table 2) indicated that heme intake was nil in the control group and all of the heme-supplemented groups consumed ~9 μmol/d. Obviously, fecal heme excretion was low in the control group. However, fecal heme excretion was also low in the heme group and in the heme plus chlorophyllin groups, indicating intensive catabolism of heme into other, nonporphyrin compounds even in the presence of chlorophyllin. In contrast, when chlorophyllin was added to the heme diet >50% of the heme was detected as such in the feces, showing that natural chlorophyll inhibits conversion or catabolism of the heme. Control experiments with heme added to feces of chlorophyllin-fed rats showed that chlorophyllin did not interfere in the assay (data not shown).

Subsequently, we measured TBARS in fecal water to examine whether heme-catalyzed lipid peroxidation in gut lumen could be inhibited by chlorophyllin and chlorophyll. TBARS increased 1.5-fold in fecal waters of the heme group compared with the control group (Fig. 4). TBARS in the heme plus chlorophyllin groups were similarly increased 1.5- to 2-fold, indicating no protective effects. In contrast, adding chlorophyllin to the heme diet reduced formation of lipid radicals. To validate the assay, we also measured TBARS formation after adding heme in the assay or supplementing control fecal water with heme. No significant increases in TBARS were observed (data not shown).

Finally, we investigated whether the observed differential effects of chlorophyllin and chlorophyll were related to a difference in hydrophobicity of the molecules. Therefore, we determined the distribution coefficients of heme, chlorophyllin, and chlorophyll in octanol:water mixtures (Table 3). A high distribution coefficient indicates that a compound is very hydrophobic. The heme distribution coefficient was highest in octanol. The addition of chlorophyllin or chlorophyll reduced the heme distribution coefficient significantly. The addition of Cu-chlorophyllin (Cu-chlorophyllin) did not alter the heme distribution coefficient, indicating no interaction with the heme.

**Table 2**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control Heme</th>
<th>Heme plus Na-chlorophyllin</th>
<th>Heme plus Cu-chlorophyllin</th>
<th>Heme plus chlorophyllin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fecal intake</td>
<td>0</td>
<td>9.0 ± 0.2</td>
<td>9.1 ± 0.2</td>
<td>9.4 ± 0.3</td>
</tr>
<tr>
<td>Heme</td>
<td>0.4 ± 0.0</td>
<td>0.7 ± 0.0</td>
<td>1.1 ± 0.0*</td>
<td>0.8 ± 0.0</td>
</tr>
<tr>
<td>Catabolized heme</td>
<td>8.2 ± 0.2</td>
<td>8.0 ± 0.2</td>
<td>8.3 ± 0.2</td>
<td>4.5 ± 0.4*</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 8. * Different from heme, P < 0.05.
2 Heme intake was calculated by multiplying individual daily food intake by the heme content of the diet. Heme in the fecal samples was determined by a modified HemoQuant assay. The amount of catabolized heme was calculated by subtracting fecal heme output from daily intake values of heme.
hydrophobic. The distribution coefficients of the supplements followed the order chlorophyll > heme > Cu-chlorophyllin > Na-chlorophyllin. These results indicate that heme and chlorophyll will accumulate in a hydrophobic environment, whereas the chlorophyllins prefer a more hydrophilic environment. The low values of Na-chlorophyllin and Cu-chlorophyllin are likely due to the 3 carboxyl groups on the porphyrin ring, and the high value for chlorophyll is due to the phytol tail attached to the porphyrin ring.

### DISCUSSION

This study shows that chlorophyllins, in contrast to chlorophyll, do not inhibit the heme-induced carcinogenic effects in rats.

Heme, chlorophyll, and chlorophyllins are tetrapyrrole molecules containing a large number of conjugate double bonds within a planar ring system. Chlorophylls are the water-soluble salts of the ubiquitous pigment chlorophyll found in green plants. Chlorophyll is used in most studies as a model compound to mimic the effects of natural chlorophyll, probably because chlorophyll is unstable in solutions and insoluble in water (17,18). Several studies, and a human intervention study, described potent anticarcinogenic and anti-inflammatory effects of chlorophyllins (17,19).

The chlorophyllins supplemented to our purified heme diets showed only a negligible inhibition of the heme-induced luminal cytotoxicity. This slight inhibition did not prevent the heme-induced changes in colonic cell turnover. In contrast, chlorophyll added to this heme diet inhibited all the heme-induced changes in luminal cytotoxicity and cell turnover.

Most of the heme ingested is delivered to the large bowel (31,32). There, a variable amount is converted to a range of iron-free porphyrins such as protoporphyrin, deuteroporphyrin, and pemporphyrin as a result of bacterial action (32). However, further degradation products such as di- and tripyrroles were not identified. The heme-induced cytotoxicity observed in our experiments results from the presence of a highly cytotoxic heme metabolite. This is a lipid-soluble, covalently modified porphyrin formed in the gut lumen of rats (7,30). Table 2 shows that chlorophyll, unlike the chlorophyllins, allowed most of the heme ingested to reach the feces without modification. This indicates that chlorophyll, but not chlorophyllins, prevents intestinal heme metabolism.

Our previous work showed that the presence of the heme metabolite in the colonic lumen damages the colon surface epithelial cells and consequently increases epithelial proliferation and inhibits apoptosis in the colonic crypts (15). Spinach or an equimolar amount of chlorophyll prevented this heme-induced effect (15). We hypothesized that this is due to a “sandwich” of heme with chlorophyll molecules. As a result, chlorophyll may block the chemical reactivity of heme and thus the formation of its cytotoxic metabolite. This “sandwich” could be due to pi-pi interactions between heme and chlorophyll in a hydrophobic complex, analogous to the mechanism described by Dashwood et al. (33) for the interaction between chlorophyllins and planar aromatic compounds, such as heterocyclic amines.

The inhibition of heme-induced luminal cytotoxicity and increased colonic cell turnover by chlorophyll but not by chlorophyllins has to be explained in terms of structural differences between the molecules. One difference between the chlorophyllins and chlorophyll is the absence of a metal or the presence of copper in the center of the tetrapyrole molecule, in contrast to magnesium in chlorophyll. Passage of chlorophyll through the stomach releases its magnesium due to the acidic pH (34,35). Magnesium is a divalent element like calcium, and intake of calcium precipitates bile acids (36) and stimulates precipitation of heme (7). Wang et al. (37) suggested that supplementation of magnesium in the diet might also precipitate bile acids. An increased magnesium concentration in the intestines might therefore precipitate heme or other components from the fecal matrix. Therefore, we adjusted the Na-chlorophyllin–supplemented diet with a concentration of magnesium equimolar to that in the chlorophyll diet. This was a minor (5.5%) increase in magnesium because of the high concentration of magnesium already present in the control (38) diet. Na-chlorophyllin supplementation to the heme diet caused only a minor inhibition of the heme-induced luminal cytotoxicity. Furthermore, no inhibitory effects on other colonic markers were observed, indicating that the magnesium of chlorophyll is not responsible for its inhibition of the heme-induced cell turnover.

We showed previously that the detrimental effects of heme coincide with heme-catalyzed lipid peroxidation in the gut lumen (7) and that both are inhibited by dietary antioxidants (12). This implies that heme has to be in close contact with fatty acids in the hydrophobic phase of the luminal contents. We now show that, in contrast to chlorophyllins, the addition of chlorophyll to a heme diet decreased this heme-induced lipid peroxidation (Fig. 4). This indicates that the chlorophyllins we used in our model cannot “sandwich” heme to inhibit a reaction between heme and fatty acids in the diet. Only chlorophyll might be able to “sandwich” heme and as a consequence inhibit the catalytic activity of heme in the generation of lipid hydroperoxides and the formation of a cytotoxic heme metabolite (39). The difference in hydrophobic behavior of chlorophyll and chlorophyllin is a consequence of a structural difference. Chlorophyll is extremely hydrophobic due to the presence of a phytol tail, which is retained during intestinal passage (35,40). On the other hand, chlorophyllins are hydrophilic as a result of the removal of the phytol tail from chlorophyll. A preference of heme and chlorophyll for a hydrophobic environment was confirmed by their high octanol/water distribution coefficients (Table 3). The lower distribution coefficient of the chlorophyllins reflects their more hydrophilic character. Finally, heme and chlorophyllins are negatively charged under physiologic conditions such as prevail in the intestines, whereas chlorophyll remains a neutral molecule. These negative charges cause repulsive forces between heme and chlorophyllin, which may prevent formation of a complex between these molecules.

In summary, our data show that the heme-induced detrimental effects were inhibited only by natural chlorophyll and not by water-soluble chlorophyll derivatives. Extrapolation of these results to humans suggests that dietary protection against

### TABLE 3

<table>
<thead>
<tr>
<th>Dietary supplement</th>
<th>Distribution coefficient</th>
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<tr>
<td>Heme</td>
<td>9.0 ± 1.7</td>
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<tr>
<td>Na-chlorophyllin</td>
<td>0.5 ± 0.0</td>
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<tr>
<td>Cu-chlorophyllin</td>
<td>1.9 ± 0.2</td>
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<td>Chlorophyll</td>
<td>162.2 ± 12.1</td>
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</table>

1 Values are means ± SEM, n ≥ 5. Distribution coefficients were determined by measurement of the distribution of the components between octanol and water.
the increased risk of colon cancer due to high consumption of red meat can be offered only by consumption of green vegetables, and not by chlorophyllin supplements.

ACKNOWLEDGMENT

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LITERATURE CITED