JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Sodium Copper Chlorophyllin: In Vitro Digestive Stability and Accumulation by Caco-2 Human Intestinal Cells

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Sodium copper chlorophyllin (SCC), a mixture of water-soluble chlorophyll derivatives, is used as both a food colorant and a common dietary supplement. Although the potential antimutagenic and antioxidant properties of this commercial preparation have been demonstrated, limited information is available on its digestion and absorption by humans. Stability of SCC was examined during simulated gastric and small intestinal digestion. Three preparations were subjected to in vitro digestion: SCC in water, SCC in water + 10% corn oil, and SCC in applesauce. SCC components from raw material preparations and in digested samples were analyzed by $C_{18}$ HPLC with photodiode array detection. Cu(II)chlorin $e_4$, the major chlorin component of SCC, was relatively stable during simulated digestion. In contrast, greater than 90% of Cu(II)chlorin $e_6$ was degraded to undetermined products during digestion. Recovery of Cu(II)chlorin $e_6$ after digestion was increased by incorporation of SCC into applesauce, suggesting a protective role of the inclusion matrix for stabilization of labile SCC components. Accumulation of SCC derivatives was investigated by using differentiated cultures of the TC7 clone of the Caco-2 human intestinal cell line. Cellular accumulation from media containing 0.5 to 60 ppm SCC was linear with intracellular content ranging between 0.2 and 29.6 $\mu$g of total SCC per mg of cellular protein. Uptake of SCC by Caco-2 cells was significantly ($p < 0.01$) lower in cultures incubated at 4 °C than in those incubated at 37 °C. Although intracellular SCC was transported into both apical and basolateral compartments when Caco-2 cells were grown on inserts, apical efflux was significantly greater ($p < 0.01$) than basolateral efflux. Stability of Cu(II)chlorin $e_4$ during in vitro digestion and effective uptake by Caco-2 enterocyte-like cells support the likelihood that a portion of this SCC component or its metabolites is absorbed from the human intestine.

**KEYWORDS: Sodium copper chlorophyllin; in vitro digestion; Caco-2 cells; cellular accumulation**

## INTRODUCTION

Increased fruit and vegetable consumption has been associated with a decreased risk in the development of chronic diseases such as cancer and cardiovascular disorders (*1*−*3*). This has resulted in intense efforts to identify physiologically active compounds in plant foods (*4*−*6*). Chlorophyll is the most ubiquitous of all natural pigments and functions as the primary photosynthetic pigment of all green plants (*7*). Sodium copper chlorophyllin (SCC) is a bright green mixture derived from natural chlorophyll that is being used increasingly as both a food supplement and colorant. Although approved for use as a food colorant in the European Community and Japan, it has met with limited use in the United States. However, a petition has recently been filed with the U.S. Food and Drug Administration for its application in dry beverage mixes. Commercial-grade SCC is prepared from a crude chlorophyll extract by reaction with methanolic sodium hydroxide followed by a replacement of the central magnesium atom with a heavy metal such as copper (*8*). The final mixture is composed of numerous chlorin compounds (**Figure 1**) derived from natural chlorophyll and includes two main components referred to as Cu(II)chlorin $e_4$ and Cu(II)chlorin $e_6$ (*9*,*10*).

A wide range of health benefits has been reported for SCC. In addition to the antiinflammatory, deodorizing, and erythropoietic activities reported almost 50 years ago (*8*), SCC also exhibits potent antimutagenic activity against a variety of known dietary and environmental mutagens such as aflatoxins, benzo[*a*]pyrene, and heterocyclic amines (*11*−*14*). Cu(II)chlorin $e_4$ and Cu(II)chlorin $e_6$ in commercial preparations of SCC also have been identified as potent antioxidants that minimize lipid peroxidation (*15*, *16*).

In contrast to numerous reports about the apparent health-promoting benefits of SCC, there is minimal information about the absorption and metabolism of these compounds in humans.

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10.1021/jf010869g CCC: $22.00 
Published on Web 02/16/2002

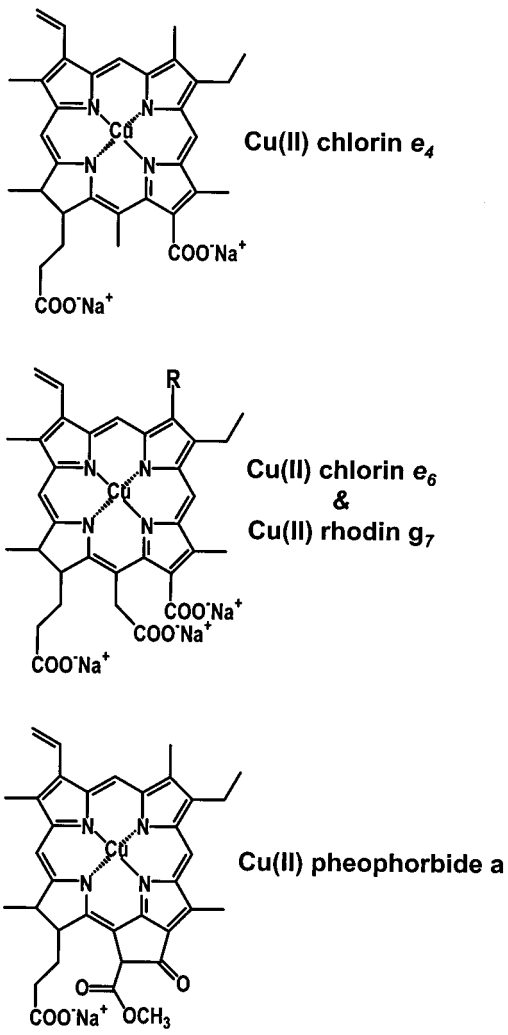


**Figure 1.** Structure of main sodium copper chlorophyllin derivatives. For Cu(II)chlorin $e_6$, R = $CH_3$; for Cu(II)rhodin $g_7$, R = CHO.

The instability of natural chlorophylls to acid, heat, and light is well established (*17*). We recently reported that native Mg-chlorophylls are completely converted to metal free pheophytin derivatives during digestion in vitro. Lipophilic pheophytin derivatives that were micellarized during the small intestinal phase of the in vitro digestion process were subsequently accumulated by the Caco-2 human intestinal cell line (*18*). The recent identification of Cu-chlorin $e_4$ and a Cu-chlorin ethyl ester in serum of human subjects ingesting SCC daily provides the first evidence that SCC can be absorbed by humans (*19*, *20*). However, the digestive stability and the efficiency of SCC absorption in humans have not been investigated.

The primary objectives of the current investigation were to evaluate the stability of SCC during in vitro digestion, and to examine SCC uptake from the luminal compartment by using monolayers of the Caco-2 cell line. This human intestinal cell line exhibits enterocyte-like characteristics (*21*), and has been validated as a general predictor of intestinal absorption in humans for numerous lipophilic drugs (*22*, *23*). This model also has been successfully utilized to investigate the uptake of nutrients such as iron (*24*, *25*) and various phytochemicals including micellarized carotenoids (*26*, *27*) and natural chlorophylls (*18*).

## MATERIALS AND METHODS

**Chemicals and Standards.** Extraction and chromatography solvents, methanol, ethyl acetate, diethyl ether, and water were certified HPLC- and ACS-grade (Fisher Chemical, Fair Lawn, NJ). Phosphate buffered saline was prepared in HPLC-grade water and acidified to pH 3 by titration with 5.0 N HCl. Commercial-grade sodium copper chlorophyllin was purchased form Sigma-Aldrich (St. Louis, MO). Purity of commercial SCC was calculated to be 47.8% based on 4.5% copper content as specified by the manufacturer with respect to the main component Cu(II)chlorin $e_4$ (MW = 644.3). This is similar to previously published values for commercial-grade SCC (*9*). Final experimental SCC concentrations were adjusted in accordance with the level of purity. Authentic standards of Cu(II)chlorin $e_4$, Cu(II)chlorin $e_6$, and Cu(II)-pheophorbide *a* were purchased from Frontier Scientific (Logan, UT) and determined to be >95% pure by HPLC analysis.

**Assessment of Digestive Stability.** Stability of SCC to human digestion was assessed by using an in vitro protocol as described originally by Garrett et al. (*26*). Three test preparations were prepared from an aqueous stock solution of SCC. The first preparation simply contained aqueous SCC (W). The second preparation consisted of a homogenized mixture of SCC and 10% corn oil (wt/wt) in water (WCO). The third preparation contained SCC homogenized in an applesauce matrix with 10% corn oil (AS). Final SCC concentrations ranged from 50 to 250 $\mu$g/mL for all experimental preparations. Representative aliquots of each preparation were subjected to a two-phase in vitro digestion protocol designed to mimic the in vivo process. The gastric phase included acidification of the sample to pH 2 with 100 mM HCl and exposure to porcine pepsin (3 mg/mL) with incubation at 37 °C for 1 h in a shaking water bath (95 rpm). The small intestinal phase was initiated by neutralizing the gastric phase with $NaHCO_3$, adding porcine pancreatin (0.4 mg/mL), lipase (0.2 mg/mL), and bile extract (2.4 mg/mL), and adjustment of final pH to 7.0 with 1 N NaOH prior to incubation at 37 °C in a shaking water bath (95 rpm). After 2 h, aliquots of the digesta were centrifuged at 167,000*g* at 4 °C for 35 min (Beckman model L7-−65 Ultracentrifuge) to separate the aqueous micellar fraction from residual solids and oil. Isolated aqueous fractions were filtered (0.2-micron pore size) to remove contaminating aggregates. All procedures were performed in subdued light, and samples were blanketed with nitrogen to minimize oxidation. Aliquots of raw material, digesta, and aqueous fraction were collected and stored under nitrogen at −80 °C until analysis.

**Accumulation of SCC by Intestinal Cells.** Caco-2 human intestinal cells were used to characterize the accumulation of SCC. The TC7 clone of Caco-2 was kindly provided by Dr. Monique Rousset, INERM, Cedex, France. Stocks and test cells were maintained as described by Han et al. (*24*) with complete medium containing high-glucose Dulbecco Modified Eagle Media (DMEM) (Sigma-Aldrich; St. Louis, MO), 10.0% heat-inactivated fetal bovine serum, nonessential amino acids (10 mL/L), L-glutamine (2.0 mmol/L), amphotericin B (0.5 mg/L), gentamicin (5.0 mg/L), HEPES (15 mmol/L), and sodium bicarbonate (44 mmol/L), in a humidified atmosphere of air/$CO_2$ (95:5) at 37 °C. Test cells (passages 75−80) were grown and differentiated in 6-well plastic dishes. Highly differentiated monolayers were used for experiments 11−14 days after reaching confluency. Prior to initiating experiments, monolayers were washed twice with 2 mL basal DMEM. Six test media were prepared by solubilizing SCC in basal DMEM at concentrations of 0.5, 1.5, 6.0, 15.0, 25.0, and 60.0 ppm. After filtration (0.2-$\mu$m pores), test media (2 mL) at either 4 or 37 °C were added to monolayers. Test media were removed at 0.25−4 h later, and monolayers were washed twice with ice-cold phosphate buffered saline (PBS). Cells were collected in 1.0 mL of ice-cold PBS (pH 7.4), centrifuged (500*g* for 5 min), and the pellet was blanketed with $N_2$ and stored at −80 °C until analysis.

Characterization of the efflux of accumulated SCC was investigated utilizing differentiated cultures of Caco-2 cells grown on BD Falcon cell-culture inserts with 0.4-$\mu$m pores (Becton Dickinson; Franklin Lakes, NJ). Intact monolayers were incubated with cDMEM containing phenol red (500 $\mu$mol/L) and 30 ppm SCC. After 4 h, inserts were washed with phenol-free cDMEM before transfer to new wells. Fresh cDMEM medium with and without 500 $\mu$mol/L phenol red was added to the insert (apical compartment) and the well (basolateral compartment), respectively. Both apical and basolateral media were collected at 4 and 20 h post loading and centrifuged at 300*g* for 5 min to remove any debris, and the supernatants were stored under a blanket of nitrogen at −80 °C until analysis. Monolayers were washed twice with ice-

cold PBS and cells were collected either after the 4 h exposure to SCC, or 4 h and 20 h post-loading. Monolayer integrity was assessed by monitoring the transfer of the phenol red from the apical to the basolateral compartment. Monolayers utilized for transport studies exhibited apical to basolateral flux of phenol red of less than 0.0002%/h/cm$^2$.

**Extraction of SCC**. Extraction of SCC components from 1 to 4 mL of the digesta and the aqueous fraction was initiated by homogenization with an equal volume of acidified PBS (pH = 3.0). Reduction of the pH ensures protonation of SCC derivatives which facilitates their extraction into organic solvent. SCC components were then extracted by the addition of 4.0 mL of diethyl ether (0.1% BHT) and vortexed for 1 min. Samples were then centrifuged (2,000*g*) for 2 min to hasten phase separation for collection of the diethyl ether layer. Extraction was repeated three times, and the combined diethyl ether fractions were dried under a stream of nitrogen, redissolved in methanol, and immediately analyzed as described.

Extraction of SCC from Caco-2 cells was initiated by addition of 200 $\mu$L of protease solution (10 mg protease per mL PBS) to resuspended cell pellet followed by incubation at 37 °C. After 30 min, 0.5 mL of acidified PBS was added, and the sample was vortexed for 1 min. SCC components were extracted into 0.5 mL of diethyl ether (0.1% BHT). Samples were vortexed and briefly centrifuged (2,000*g*) to separate the phases. The diethyl ether layer was collected, and extractions were repeated three times. Combined fractions of diethyl ether were dried under a stream of nitrogen and analyzed immediately.

**SCC Analysis.** High-performance liquid chromatography (HPLC) of chlorophyllin derivatives was performed with a Hewlett-Packard model 1100 HPLC system equipped with a model 1100 diode array detector (Santa Clara, CA). A Vydac 201TP54 analytical scale (4.6 mm i.d. × 250 mm) $C_{18}$ reversed-phase column (Hesperia, CA) with a $C_{18}$ stationary phase guard column was used. Separations were achieved by gradient elution with a binary mobile phase of methanol−water−acetic acid in reservoir A (75:24.5:0.5) and ethyl acetate in reservoir B. Initial conditions were set at 100% A with a linear gradient to 50:50 A:B over 20 min, followed by a 5.0 min linear gradient back to 100% A for a final chromatographic run time of 25 min. Detection and tentative identification of major SCC components were accomplished by co-chromatography with authentic standards, and comparison of electronic absorption spectra obtained from in-line diode array detection with previously published electronic absorption spectra of major SCC derivatives (*28*).

**Data Analysis**. The quantity of SCC derivatives recovered in digesta and aqueous fractions relative to that in the raw material is defined as percent recovery. All data were analyzed with StatView 5.0 (SAS Institute, Cary, NC). Descriptive statistics including mean and standard error of mean (SEM) were calculated for each SCC derivative's percent recovery and Caco-2 intracellular content from a minimum of 3−5 independent measurements. Group differences were determined by analysis of variance by using Fisher's PLSD post-hoc test ($\alpha < 0.05$).

## RESULTS AND DISCUSSION

**HPLC Analysis.** A representative HPLC chromatogram of commercial-grade SCC is shown in **Figure 2**. This material contained numerous copper chlorophyllin complexes. Four derivatives were tentatively identified based on co-chromatography with authentic standards and comparison of electronic absorption spectra to published values (*28*). As shown in **Table 1**, Cu(II)chlorin $e_4$ (81%) was the major component, followed by Cu(II)chlorin $e_6$ (10%), Cu(II)rhodin $g_7$ (3%), and Cu(II)-pheophorbide *a* (1%). These results are comparable to those in previously published reports with expected differences arising from lot-to-lot variability (*9*, *28*). A number of other chlorin components were detected and observed to have spectral properties similar to those of the main derivatives. These likely represent isomers and/or minor degradation products of the parent compounds. The described chromatographic method facilitates rapid and selective separation of major components from commercial-grade SCC.

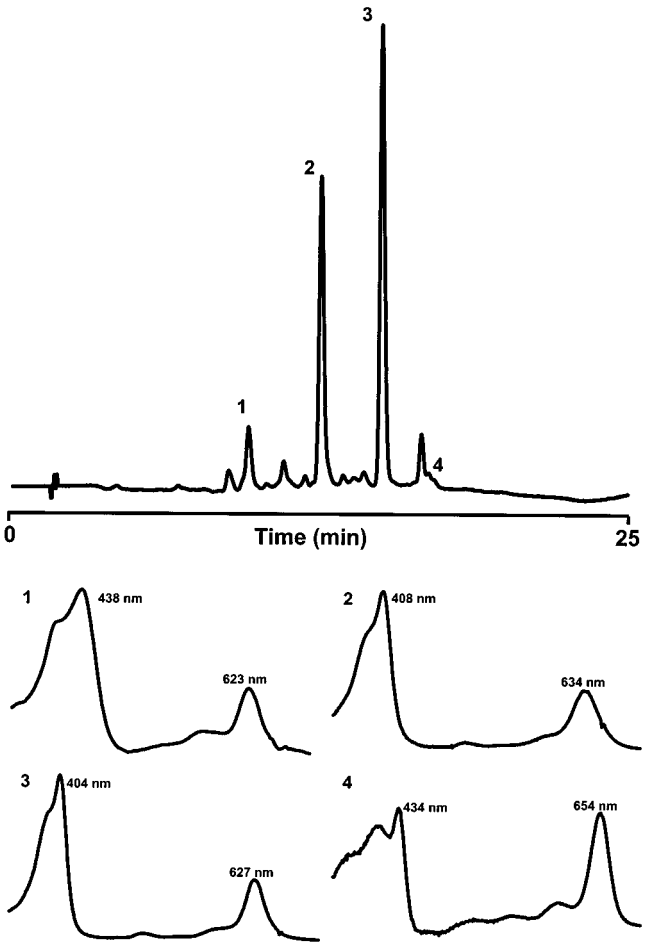


**Figure 2.** HPLC separation of predominant derivatives of commercial-grade sodium copper chlorophyllin. Peak identifications: 1, Cu(II)rhodin $g_7$; 2, Cu(II)chlorin $e_6$; 3, Cu(II)chlorin $e_4$; 4, Cu(II) pheophorbide *a*. Ultraviolet and visible absorption spectra were collected online by diode array detection between 350 and 700 nm.

**Table 1.** Absorption Maxima of Main SCC Components Obtained by Online Photodiode Array Detection from a C18–HPLC Separation

| | observed $\lambda_{max}$ (nm) | | published[a] $\lambda_{max}$ (nm) | |
|---|---|---|---|---|
| compound | Soret band | Q band | Soret band | Q band |
| Cu(II) pheophorbide *a* | 402, 424 | 654 | 401, 424 | 653 |
| Cu(II) chlorin $e_6$ | 408 | 634 | 407 | 633 |
| Cu(II) chlorin $e_4$ | 404 | 627 | 404 | 626 |
| Cu(II) rhodin $g_7$ | 438 | 623 | 436 | 622 |

[a] Inoue et al., 1994 (*28*).

**Stability of SCC to In Vitro Digestion.** Stability of SCC during simulated digestion was examined with an in vitro model that simulates both gastric and small intestinal phases of digestion. SCC was incorporated at 50 to 250 $\mu$g per g into three separate matrixes designed to mimic typical consumption including applesauce (AS), water (W), and a water−corn oil emulsion (WCO). The chromatographic profile of chlorophyllin species in the test meal, digesta, and aqueous fraction was subsequently determined by HPLC. Chlorophyllin derivatives transferred from the starting raw material to the aqueous fraction of the digesta are assumed to be available for uptake by the intestinal cells. SCC components from the raw materials were efficiently transferred (>60%) to the aqueous fraction for all preparations, presumably because of the hydrophilicity of SCC.

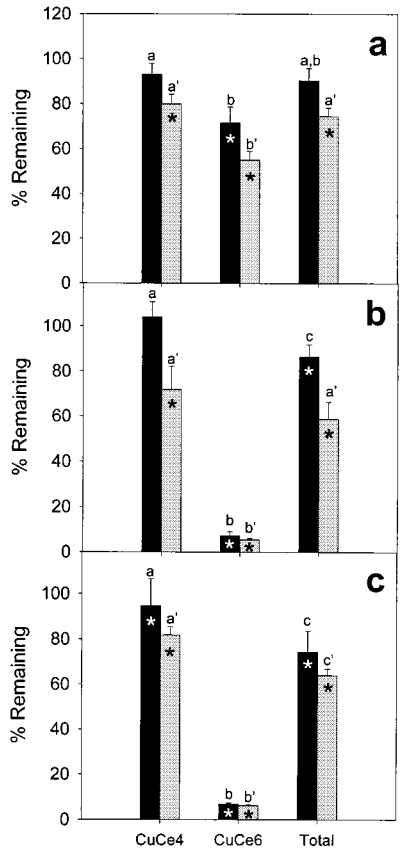


**Figure 3.** Recovery of major SCC derivatives in (■) digesta and (□) aqueous fractions from in vitro digestion of apple sauce preparation, AS (panel A); water/no lipid preparation, W (panel B); and water/lipid preparation, WCO (panel C). Data represent the percentage of SCC present in digesta and aqueous fraction of digesta relative to starting raw material, which is arbitrarily set at 100%. Digestions and analyses were performed as described in Materials and Methods. Values represent means ± SEM for 4 independent digestions. Different letters above error bars indicate that recovery of Cu(II)chlorin $e_6$ was significantly lower than that of Cu(II)chlorin $e_4$ in AS ($p < 0.05$), WCO ($p < 0.001$), and W ($p < 0.001$). Preparations for which pigment recoveries differed significantly ($p < 0.05$) from the original raw material are indicated by an asterisk (*).

Minimal differences were observed between W and WCO preparations, indicating that association of SCC with lipid-bile salt micelles is not required for transfer to the aqueous fraction as is required with natural chlorophyll derivatives (*18*). Therefore, ingestion of foods containing fat does not appear to be required to optimize intestinal cell uptake and, by extension, bioavailability of these chlorophyll derivatives.

Quantitative and qualitative changes in the chlorin content of SCC were noted after digestion of the three preparations. Cu(II)chlorin $e_4$, the main component of SCC, exhibited excellent stability with greater than 90% from the raw material present in the digesta fraction after in vitro digestion. Recovery of Cu(II)chlorin $e_4$ in the aqueous fraction was 70 to 80% for all preparations (**Figure 3**). The amounts of SCC in the aqueous fraction were consistently lower than those in digesta. This may reflect binding of porphyrins to remnant solids from crude porcine pancreatin and bile extract, as well as residual pectins and fiber in applesauce, as a green precipitate was visible following centrifugation of the digesta fraction in all three preparations.

In contrast with the relative stability of Cu(II)chlorin $e_4$, recovery of Cu(II)chlorin $e_6$ after digestion of both W and WCO preparations was only 10% ($p < 0.001$; **Figure 3**). Incorporation of SCC into applesauce significantly ($p < 0.05$) increased recovery of Cu(II)chlorin $e_6$ after digestion. The decline in Cu(II)chlorin $e_6$ relative to Cu(II)chlorin $e_4$ contributes to qualitative changes in the SCC profile observed post digestion, but does not greatly affect final SCC content because Cu(II)chlorin $e_4$ is most abundant in this commercial SCC preparation.

Degradation of chlorophyll derivatives by reactive oxygen species including singlet oxygen has been demonstrated (*29*). Penttilä et al. (*30*) reported that chlorophyllin bleaching proceeds by a peroxidative process and can be retarded by addition of reducing agents such as ascorbate. Moreover, Salin et al. (*31*) reported the selective loss of Cu(II)chlorin $e_6$ during photobleaching of crude SCC. We speculated that the preferential degradation of Cu(II)chlorin $e_6$ during the digestion occurred by oxidative processes. However, the addition of ascorbic acid to levels as high as 1 mg per g of test meal failed to improve the recovery of Cu(II)chlorin $e_6$. Therefore, the greater stability of Cu(II)chlorin $e_6$ in applesauce requires consideration of other matrix effects including buffering capacity, light protection, and nonnutritive components such as phenolic antioxidants. This protective effect will be important when preparing products supplemented with SCC that are intended for human consumption.

The stability of phytochemicals during digestion is important for their potential health benefits. In vitro studies assessing functional activity often use purified natural compounds without consideration of digestive stability and bioavailability. We recently reported that natural chlorophylls are converted to metal free pheophytins during simulated digestion, thereby generating a diverse array of metal-free chlorophyll derivatives available for uptake by human intestinal cells (*18*). Thus, the protective effects of natural chlorophyll *a* and *b* reported in the literature (*11*, *32*) would more likely reflect the activities of their metal-free pheophytin derivatives rather than the native compounds. The stability of some water-soluble copper chlorophyll derivatives such as Cu(II)chlorin $e_4$ during simulated digestion suggest that these Cu(II) complexes are the predominant species in the human intestinal lumen after ingestion of SCC. It is interesting that Egner et al. (*20*) identified both Cu(II)chlorin $e_4$ and a Cu(II)chlorin $e_4$ ethyl ester, but not Cu(II)chlorin $e_6$, in plasma of patients ingesting 300 mg of SCC daily for four months. Our results suggest that failure to detect Cu(II)chlorin $e_6$ in plasma may be due to selective degradation of this derivative in the GI tract.

**Uptake and Accumulation of SCC by Intestinal Epithelial Cells.** Cellular uptake and accumulation of SCC derivatives by enterocyte-like cells was studied with fully differentiated cultures of the TC7 clone of Caco-2 cells. SCC was solubilized in basal DMEM and serially diluted to concentrations ranging from 0.5 to 60 ppm for presentation to the apical surface of the monolayers. This range approximates concentrations in the lumen resulting from consumption of 1.0 to 100 mg of SCC, assuming that the ingested softgel or liquid SCC supplement (60−100 mg SCC per serving) will be diluted by approximately 2 L of gastric and intestinal fluids (*33*). Cells were harvested after incubation for indicated times and SCC content was determined.

SCC uptake by Caco-2 cells was investigated at various times during exposure to medium containing 30 ppm SCC for as long as 1 h (**Figure 4**). The intracellular ratio of Cu(II)chlorin $e_4$ to $e_6$ was similar to that of the test medium (data not shown) indicating that uptake of the major SCC derivatives was proportional to their concentration in the media. Uptake of Cu(II)chlorin $e_6$ by human intestinal cells in vitro further highlights

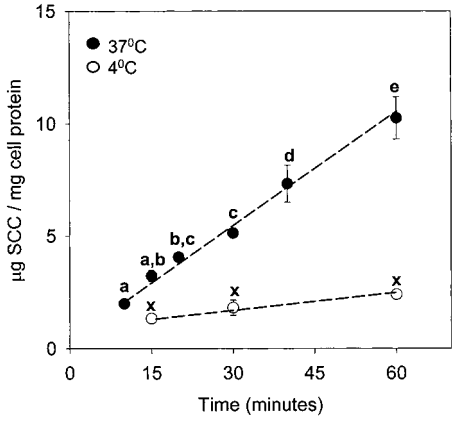


**Figure 4.** Uptake of SCC by Caco-2 human intestinal cells. Differentiated monolayers of Caco-2 cells were incubated in medium containing 30 ppm SCC. Medium was removed and monolayers washed with ice cold PBS at indicated times, and the quantity of SCC in cells was determined as described in Materials and Methods. Means ± SEM for 3–5 independent measurements are reported. The presence of different letters indicates significant ($p < 0.05$) increase in cellular SCC content with incubation time for cultures incubated at 37 °C. Total SCC content differed significantly ($p < 0.01$) between cultures incubated at 37 and 4 °C after incubation for 15, 30, and 60 min. SCC accumulation did not increase significantly ($p > 0.05$) with increased incubation time when cultures were incubated at 4 °C.

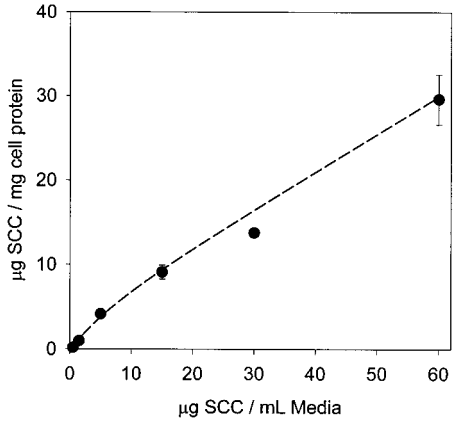


**Figure 5.** Accumulation of SCC by Caco-2 cells is proportional to medium concentration of SCC. Data represent mean ± SEM for 3–5 independent measurements of total SCC per mg of cell protein after 4 h incubation in basal DMEM containing between 0.5 and 60 ppm of SCC.

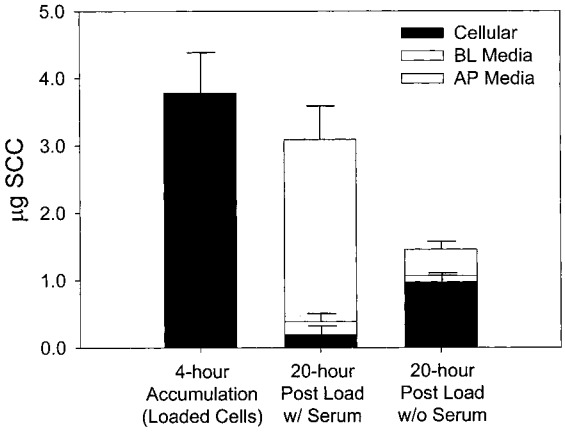


**Figure 6.** Recovery and efflux of SCC from differentiated monolayers of Caco-2 grown on inserts. Cells were preloaded by addition of cDMEM containing 30 ppm SCC to the apical compartment for 4 h. Monolayers were washed and either harvested to determine SCC content or incubated for 20 h in fresh DMEM with and without serum (present as 10% FBS) as described in Materials and Methods. Cells and apical and basolateral media were collected and analyzed for SCC to determine retention, efflux, and recovery. Data represent mean ± SEM for 3 independent measurements of total SCC in Caco-2 monolayers, apical medium, and basolateral medium after incubation of preloaded Caco-2 monolayers for 20 h in either basal DMEM (w/o serum) or cDMEM (w/ serum).

the impact of digestive instability of this chlorin derivative. Cellular content of total SCC, i.e., Cu(II)chlorin $e_4$ + $e_6$, increased linearly with length of exposure at 37 °C. After 1 h, cells contained 10.3 $\mu$g total SCC per mg cellular protein. Cellular uptake decreased by approximately 75% ($p < 0.01$) in cultures incubated at 4 °C (**Figure 4**). The accumulation of total SCC also was decreased approximately 70% ($p < 0.001$) when cultures were exposed to medium containing 1.5, 15, and 30 ppm SCC at 4 °C for 4 h (data not shown). These data suggest that SCC derivatives are transported into enterocytes by a facilitated process. Receptor-mediated transport of porphyrins has been reported. A human plasma membrane heme transporter has been characterized in both Caco-2 and HepG2 cell lines (*34*), and transferrin-mediated internalization of heme and other porphyrins was observed in murine erythroleukemia cells (*35*). Additional studies of SCC transport across the intestinal epithelium are merited, especially in light of recent isolation of SCC metabolites in human sera (*20*).

Intracellular content of SCC was proportional to the amounts in medium with cell levels ranging from 0.1 to 28 $\mu$g total SCC per mg cellular protein, after incubation in medium containing 0.5−60.0 ppm SCC at 37 °C for 4 h (**Figure 5**). Therefore, cellular accumulation of SCC was not saturated over the 120-fold range of SCC in the medium. The quantity in the cells represented 45 to 60% of SCC added to the test medium.

Stability of SCC in test media was investigated in a cell-free tissue culture environment. Media containing 30 ppm SCC test media was added to cell free wells and incubated at 37 °C for 4 h in a humidified atmosphere of air/$CO_2$ (95:5). Recovery of total SCC was 70% with 30% losses of both Cu(II)chlorin $e_4$ and $e_6$ (data not shown). There was no indication that SCC adhered to the walls of the cell culture vessel. Basal DMEM does not contain ascorbate or other water-soluble antioxidants. The fetal bovine serum added to 10% final volume contributes low levels of ascorbate, α-tocopherol, and other antioxidants. Thus, it is likely that the loss of SCC in cell-free environment reflects oxidation degradation of SCC to unknown products.

An initial assessment of SCC retention by Caco-2 cells was undertaken to determine possible metabolism and transepithelial transport of these chlorophyllin derivatives. Medium (1.5 mL) containing 30 ppm SCC was added to the apical compartment of differentiated Caco-2 monolayers grown on inserts (*24*). Caco-2 cells accumulated 6.3 ± 1.1 $\mu$g SCC per mg cellular protein after 4 h and are referred to as loaded cells. Following SCC accumulation, monolayers were washed and transferred to new wells before adding 1.5 and 2.5 mL of fresh DMEM with or without serum (10%) to the apical and basolateral chambers, respectively. Following 20 h of incubation (post load), SCC was quantified in apical and basolateral media and Caco-2 monolayers. Recovery of SCC in media plus cells was significantly ($p < 0.01$) greater when pre-loaded cells were incubated with medium with 10% serum (84.0 ± 7.7%) compared to serum-free medium (41.9 ± 9.0%) (**Figure 6**). Approximately 5 and 25% of the originally accumulated SCC was retained by Caco-2 cells after incubation in medium with and without serum, respectively. The majority (72.9 ± 8.7%) of SCC was present

in the apical compartment of cultures incubated in serum-containing medium. The apical compartment of serum-free cultures contained 9.7 ± 1.5% of the SCC originally accumulated by the Caco-2 cells during the 4 h loading. In contrast, transfer of cellular SCC to the basolateral compartment was only 2−5%. Thus, the presence of serum in the apical media was necessary for the efficient efflux and recovery of SCC in the apical compartment, suggesting that binding of SCC to proteins (e.g., albumin) may protect SCC from degradative/oxidative reactions.

Caco-2 have been shown to express apical efflux transport systems such as P-glycoprotein (*36*). Efficient apical efflux of SCC would significantly limit apical to basolateral transport, thereby decreasing absorption of SCC components. The observed transport of small amounts of SCC across the intestinal epithelia agrees with the presence of low concentrations of several SCC components in human plasma after consumption of 300 mg of SCC per day for 4 months (*19*, *20*). More detailed studies are required to explain these observations and apparently low efficiency of transfer across the basolateral membrane. These preliminary data suggest that efficient uptake of SCC by human intestinal cells is accompanied by poor retention of these compounds primarily due to apical efflux, metabolism, and perhaps oxidative degradation such as that noted in cell free media. The expression of heme oxygenase and biliverdin reductase activities in Caco-2 cells (*37*) supports the possibility that some portion of intracellular SCC may be metabolized.

In conclusion, commercial-grade SCC is composed of two prominent derivatives, Cu(II)chlorin $e_4$ and Cu(II)chlorin $e_6$. When subjected to simulated digestion, Cu(II)chlorin $e_4$ was relatively stable with greater than 70% recovery in the aqueous fraction. Conversely, the majority of Cu(II)chlorin $e_6$ in the preparation was lost during in vitro digestion. Incorporation of SCC into an applesauce matrix diminished the loss of Cu(II)-chlorin $e_6$, suggesting that an inclusion matrix can be considered for stabilizing labile SCC components following ingestion. Caco-2 human intestinal cells readily accumulated SCC when the extracellular concentration was between 0.5 and 60 ppm. Nonsaturable but temperature-dependent accumulation of SCC in Caco-2 suggest the possibility of both passive and active transport through the intestinal epithelia. The stability of Cu(II)chlorin $e_4$ during in vitro digestion, its efficient uptake, and its low but detectable transport into the basolateral compartment by differentiated monolayers of Caco-2 cells, suggest that this SCC derivative can be absorbed and transported to peripheral tissues.

## ABBREVIATIONS

SCC, sodium copper chlorophyllin; CuCe4, Cu(II)chlorin $e_4$; CuCe6, Cu(II)chlorin $e_6$; HPLC, high-performance liquid chromatography; AS, apple sauce preparation; WCO, water/lipid preparation; W, water/no lipid preparation; DMEM, Dulbecco's modified Eagles medium; PBS, phosphate buffered saline; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; BHT, butylated hydroxytoluene.

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**Received for review July 9, 2001. Revised manuscript received December 10, 2001. Accepted January 3, 2002.**

JF010869G