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Reduction of Malachite Green to Leucomalachite Green by Intestinal Bacteria

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Intestinal microfloras from human, rat, mouse, and monkey fecal samples and 14 pure cultures of anaerobic bacteria representative of those found in the human gastrointestinal tract metabolized the triphenylmethane dye malachite green to leucomalachite green. The reduction of malachite green to the leuco derivative suggests that intestinal microflora could play an important role in the metabolic activation of the triphenylmethane dye to a potential carcinogen.

The triphenylmethane dye malachite green is highly soluble in water and has long been used in the aquaculture industry as a fungicide, parasiticide, and disinfectant (1). Malachite green is also used extensively for dyeing silk, wool, jute, leather, ceramics, and cotton (7) and as a cytochemical staining agent (9). Malachite green presently is not permitted as a food coloring agent or for use in food fish in the United States; however, it is still used elsewhere in the aquaculture and seafood industries, despite the lack of approval from regulatory authorities (12). Malachite green and its reduced form, leucomalachite green, may persist in edible fish tissues for extended periods of time (2–4, 16, 21, 22). Therefore, there are both environmental and human health concerns about bioaccumulation of malachite green and leucomalachite green in terrestrial and aquatic ecosystems.

Malachite green is highly toxic to mammalian cells (6, 14, 17), at concentrations as low as 0.1 μg/ml. It also enhances liver tumor formation in rats and causes reproductive abnormalities in rabbits and fish (8, 18). Because of its low cost, effectiveness as an antifungal agent for commercial fish hatcheries, and ready availability, the general public may be exposed to this dye and its metabolites through the consumption of treated fish (8). Direct exposure to workers could also occur in the dye and aquaculture industries (7). Since malachite green is similar in structure to carcinogenic triphenylmethane dyes, it may be a potential human health hazard (10). Malachite green was recently nominated by the Food and Drug Administration as a priority chemical for carcinogenicity testing by the National Toxicology Program (7).

Intestinal microflora is important in the metabolism and enterohepatic circulation of drugs (20). The bacterial flora of the human gastrointestinal tract is complex, consisting of over 400 species, with 10^{11} to 10^{13} bacteria per g of feces (13). Any compound taken orally or entering the intestine via the biliary tract, the bloodstream, or secretion directly into the lumen is a potential substrate for metabolism by the intestinal microflora (5). Therefore, we studied the metabolism of malachite green by rat, mouse, monkey, and human intestinal microfloras and by pure cultures of anaerobic bacteria (American Type Culture Collection [ATCC], Rockville, Md.) common to the gastrointestinal tract.

Several anaerobic bacteria commonly found in the human gastrointestinal tract (Table 1) reduced malachite green (Chemsyn Science Laboratories, Lenexa, Kans.). *Clostridium perfringens*, *Escherichia coli*, and *Peptostreptococcus anaerobius* converted almost all of the dye to the leuco derivative. *Bacteroides fragilis*, *Enterococcus faecalis*, *Fusobacterium prausnitzii*, *Peptostreptococcus magnus*, *Peptostreptococcus productus*, *Ruminococcus albus*, and *Ruminococcus flavofaciens* metabolized more than 30% of the malachite green added. *Bacteroides thetaiotaomicron*, *Bifidobacterium adolescentis*, a *Citrobacter* sp., and *Lactobacillus acidophilus* metabolized significantly less malachite green. Each of the cultures of human, rat, mouse, and monkey intestinal microfloras converted virtually all of the dye to its leuco derivative.

High-pressure liquid chromatography (HPLC) analysis (15) with photodiode array detection was used to monitor conversion of malachite green by rat intestinal microflora to leucomalachite green. A chromatogram of an 8-h incubation is shown in Fig. 1. A major peak eluting at 3.45 min coeluted with authentic leucomalachite green and had UV-visible spectral

TABLE 1. Metabolism of malachite green by intestinal microflora

Organism(s)[a] or sample tested	% Conversion to leucomalachite green[b]
Bacteroides fragilis ATCC 23745	30.2
Bacteroides thetaiotaomicron ATCC 29148	16.3
Bifidobacterium adolescentis ATCC 15703	24.4
Citrobacter sp. strain ATCC 25405	12.3
Clostridium perfringens ATCC 3624	99.3
Enterococcus faecalis ATCC 19433	55.0
Escherichia coli ATCC 25922	99.0
Fusobacterium prausnitzii ATCC 27768	63.4
Lactobacillus acidophilus ATCC 332	7.3
Peptostreptococcus anaerobius ATCC 27337	99.0
Peptostreptococcus magnus ATCC 14955	52.7
Peptostreptococcus productus ATCC 27340	41.1
Ruminococcus albus ATCC 27210	52.0
Ruminococcus flavofaciens ATCC 19208	35.0
Human intestinal microflora[c]	99.0
Rhesus monkey intestinal microflora[c]	99.0
C3H/HEN-MTV mouse intestinal microflora[c]	100.0
Fischer 344 rat intestinal microflora[c]	100.0
Control (sterile medium plus malachite green)	0.0

[a] Cultures were incubated with 300 μg of malachite green in 5 ml of brain heart infusion broth (Carr-Scarborough, Decatur, Ga.) for 24 to 48 h under anaerobic conditions.

[b] The cultures and media were analyzed directly by HPLC (15).

[c] Fecal samples were obtained from freshly voided feces collected in sterile specimen containers (11).

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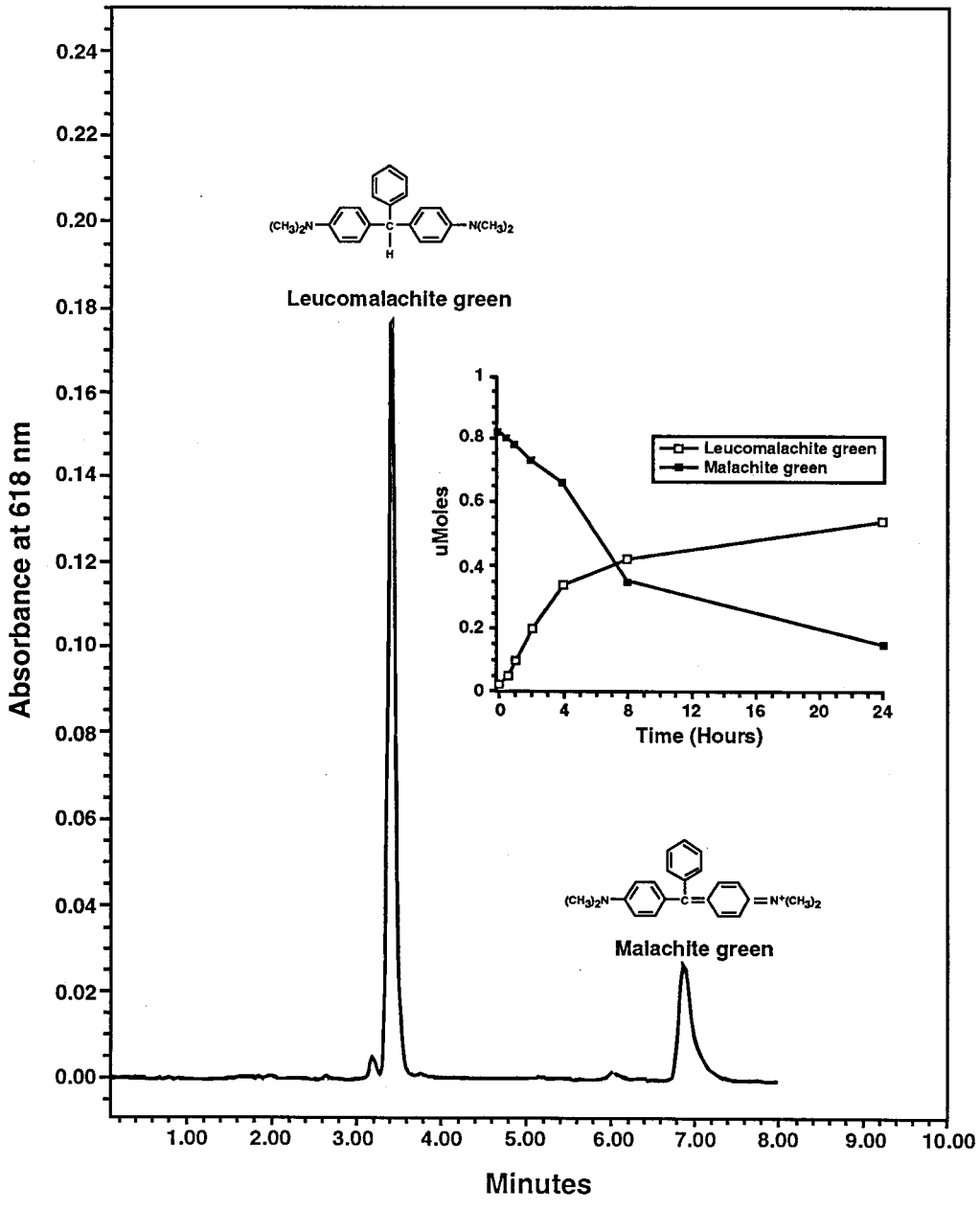

FIG. 1. HPLC elution profile showing conversion of malachite green to leucomalachite green by rat intestinal microflora. (Inset) Disappearance of malachite green and subsequent appearance of leucomalachite green in cultures of rat intestinal microflora.

properties identical to those of the authentic compound. Malachite green eluted at 6.85 min. More than 50% of the dye was converted to the leuco derivative within 8 h of incubation, with 84% conversion after 24 h (Fig. 1 [inset]). A cell-free control with malachite green showed no conversion in 24 h.

A diethyl ether extract from a culture of *C. perfringens* dosed with malachite green was analyzed by HPLC-electrospray ionization mass spectrometry (MS). For HPLC-MS analysis, a VYDAC RP18 Pharmaceutical Column (25 cm by 2.1 mm, 5-μm particle size) (Vydac, Hesperia, Calif.) heated at 40°C was used. The mobile phase was 40% methanol in H_2O (0.1% formic acid) for 2 min followed by a 10-min linear gradient to 95% methanol (0.1% formic acid), where it was held for an additional 8 min with a flow rate of 0.35 ml/min. Mass spectral analyses were performed by using a Hewlett-Packard 5989 quadrupole mass spectrometer (Hewlett-Packard Co., Wilmington, Del.) operated under standard positive-ion electrospray conditions. The main metabolite formed from malachite green eluted at 9 min, as shown in the total-ion chromatogram (Fig. 2A). An extracted-ion chromatogram for *m/z* 331 is also shown (Fig. 2B). The positive-ion electrospray mass spectrum of the metabolite (Fig. 2C) shows the protonated molecule $(M+H)^+$ at *m/z* 331 and two weak ions at *m/z* 166 and 159. Although electrospray ionization produces few ions for characterization, authentic leucomalachite green (Aldrich Chemical Co., Milwaukee, Wis.) yielded identical data (not shown) when analyzed under similar conditions.

This investigation supports the observation of Singh et al.

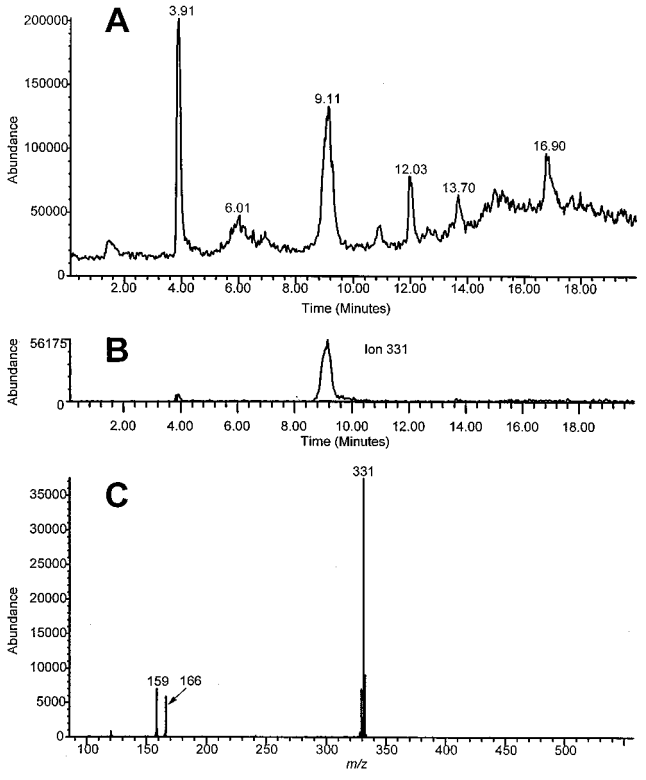

FIG. 2. Positive-ion electrospray mass spectral analysis of an extract from a culture of *C. perfringens* dosed with malachite green. (A) Total-ion chromatogram; (B) extracted-ion chromatogram for *m/z* 331, representing leucomalachite green; (C) positive-ion electrospray mass spectrum of the metabolite at a t_R of 9.11 min.

(19) that rat cecal contents metabolized malachite green to a fluorescent compound. We found that the reduction product of malachite green produced by intestinal microflora was its leuco derivative. Leucomalachite green was identified by HPLC-MS and UV-visible spectral properties which were comparable to those of the authentic compound. This finding also corresponds with our earlier study with the structurally similar triphenylmethane dye gentian violet, which was reduced to leucogentian violet (11). The extent of conversion of malachite green to leucomalachite green by rat, monkey, and human intestinal microfloras was 99 to 100% in all replicates. Since leucomalachite green is structurally similar to the leuco forms of other carcinogenic triphenylmethane dyes (10), the enzymatic reduction of malachite green to leucomalachite green by intestinal microflora could play a critical role in metabolic activation to a potential carcinogen. Studies of the carcinogenicity of malachite green and its leuco form are now being conducted.

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