Identification of S-(n-Butylcarbamoyl)glutathione, a Reactive Carbamoylating Agent, as a Biliary Metabolite of Benomyl in the Rat†

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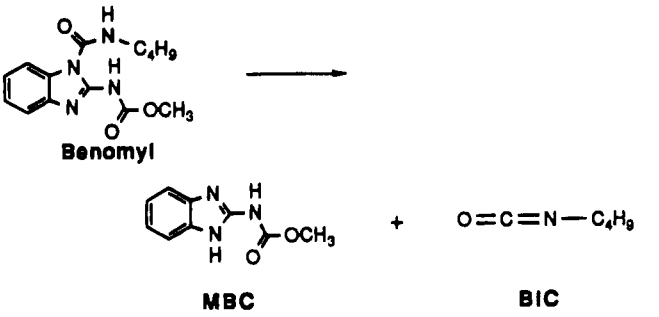
Treatment of rats with benomyl [methyl 1-(n-butylcarbamoyl)-2-benzimidazolecarbamate; 100 mg kg^{-1} ip] led to the detection of a novel biliary metabolite of this widely used systemic fungicide. By means of on-line liquid chromatography–tandem mass spectrometry, this metabolite was identified as a carbamoylated derivative of glutathione, *viz.* S-(n-butylcarbamoyl)glutathione (SBuG), whose excretion in bile over 4.5 h accounted for *ca.* 1% of the dose. *In vitro* experiments demonstrated that SBuG and the corresponding cysteine adduct exhibited carbamoylating activity toward the thiol groups of free cysteine and glutathione when the elements of n-butyl isocyanate were transferred to the acceptor nucleophiles. SBuG also proved to be highly cytotoxic to isolated rat hepatocytes at a concentration of 1 mM. The results of this study raise the possibility that SBuG may serve as a latent form of n-butyl isocyanate *in vivo* and thereby mediate some of the adverse effects of benomyl.

Keywords: *Benomyl; metabolism; toxicity; butyl isocyanate; glutathione conjugation; liquid chromatography–tandem mass spectrometry*

INTRODUCTION

Benomyl [methyl 1-(n-butylcarbamoyl]-2-benzimidazolecarbamate] has been employed widely as a systemic fungicide for broad-spectrum application on food crops and ornamental plants. Recently, however, the use of a dry flowable formulation of benomyl, Benlate DF, has been linked to crop damage in several southern regions of the United States, and there have been claims of adverse effects in animals and humans exposed to this agent (Winter and Teaf, 1993). Benomyl is known to affect the male mammalian reproductive system (Hess *et al.*, 1991, and references cited therein; Linder *et al.*, 1992) and to be embryotoxic, as well as teratogenic (Cummings *et al.*, 1992). However, the biochemical basis for these reported ill-effects remains unclear. Chemical studies have shown that benomyl degrades in both aqueous (Tang *et al.*, 1992) and organic solutions (Chiba and Cherniak, 1978) to methyl benzimidazolecarbamate (MBC, considered to be the active fungicide) and butyl isocyanate (BIC) (Scheme 1). Although the majority of attention has focused on MBC in terms of its toxicity and metabolism in animals and plants (Somerville, 1986), the high chemical reactivity and well-known toxic effects of BIC (Bayer, 1992; Chiba *et al.*, 1987) highlight the need for further information on the potential release of this isocyanate in mammals exposed to the parent fungicide. Interestingly, in the course of early metabolism studies in which radiolabeled benomyl was administered to rats, urine samples were found to contain N-acetyl-S-(n-butylcarbamoyl)cysteine (Axness and Fleeker, 1979). This observation suggested that BIC may indeed be released from benomyl *in vivo* and that this reactive intermediate undergoes metabolism via conjugation with glutathione (GSH) in a fashion similar to that established for related isocyanates, such as methyl and 2-chloroethyl isocyanate (Pearson *et al.*, 1990; Davis *et al.*, 1993). In this paper, we report on the identification and quantitative analysis of S-(n-butylcarbamoyl)glutathione (SBuG, Scheme 2), the GSH conjugate of BIC, in the bile of rats dosed with benomyl. In addition, we demonstrate that SBuG exhibits comparable cytotoxicity toward isolated hepatocytes as benomyl itself and that both SBuG and the corresponding cysteine adduct, S-(n-butylcarbamoyl)cysteine (SBuC), act as carbamoylating agents *in vitro* and thus may represent latent forms of BIC *in vivo*.

Scheme 1. Degradation of Benomyl to MBC and BIC



MATERIALS AND METHODS

Materials. Benomyl was a gift from DuPont Agricultural Products (Wilmington, DE). n-Butyl isocyanate was purchased from the Aldrich Chemical Co. (Milwaukee, WI), while GSH (reduced form) was from Sigma Chemical Co. (St. Louis, MO). Polyethylene 10 (PE-10) tubing was obtained from Becton Dickinson & Co. (Parsippany, NJ), and all other solvents and chemicals were of HPLC or reagent grade and were used as received. The S-linked conjugates SBuG, SBuC, and S-(n-propylcarbamoyl)glutathione (SPrG; employed as the internal standard for quantitative analysis of SBuG) were obtained by synthesis, as outlined below.

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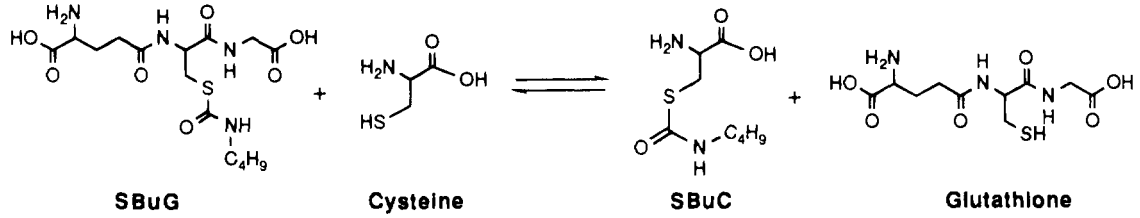
Scheme 2. Structures and Thiol Exchange Reactions of SBuG and SBuC



Instrumentation. ^{1}H NMR spectra were recorded at 300 MHz on a Varian VXR-300 spectrometer (Varian Association Inc., Palo Alto, CA). Samples were analyzed in D_2O and chemical shifts are reported in ppm (δ) downfield from 3-(trimethylsilyl)-[*2,2,3,3-d*$_4$]propionic acid sodium salt (0.00 ppm). Tandem mass spectrometry (MS/MS) and on-line liquid chromatography–tandem mass spectrometry (LC–MS/MS) were carried out on a Sciex API III triple-quadrupole mass spectrometer equipped with an Ionspray source, as described previously (Davis *et al.*, 1993). LC–MS/MS analyses employed a narrow bore (150 × 2 mm i.d.) C_{18} column coupled to the mass spectrometer via a splitting tee (Davis *et al.*, 1993). Conventional reversed phase HPLC separations were performed on a Shimadzu Model LC-10AD twin-pump system, equipped with a gradient controller and a Shimadzu Model SPD-10A variable-wavelength UV detector set to monitor absorption at 214 nm. An Altex ODS column (250 mm × 4.6 mm i.d., 5 μm; Beckman, Berkeley, CA) and an Ultrasphere ODS column (250 mm × 10 mm i.d., 5 μm; Beckman) were employed, respectively, for analytical and semipreparative work. A binary mobile phase consisting of 12% acetonitrile [containing 0.1% trifluoroacetic acid (TFA)] in water (0.1% in TFA) was utilized for both types of separations.

Synthesis of SBuG. To a two-neck 50 mL flask containing GSH (184 mg, 0.6 mmol) in a solution (12 mL) of tetrahydrofuran and water (2:1 v/v) was added dropwise BIC (0.4 mL, 4.5 mmol) at room temperature under argon with vigorous stirring. The mixture was allowed to stir for an additional 15 min, following which the volatile components were evaporated under reduced pressure and the water was removed by lyophilization. The crude product was purified by HPLC to afford SBuG (75 mg, 31%): ^{1}H NMR δ 0.86 (t, J = 7.3 Hz, 3H, $NC_3H_6CH_3$), 1.11–1.35 (m, 2H, $NC_2H_4CH_2CH_3$), 1.38–1.50 (m, 2H, $NCH_2CH_2C_2H_5$), 2.10–2.22 (m, 2H, Glu-β,β'), 2.52 (t, J = 6.9 Hz, 2H, Glu-γ,γ'), 3.10–3.24 (m, 3H, Cys-β and $NCH_2C_3H_7$), 3.81 (dd, J = 14.6 and 5.0 Hz, 1H, Cys-β'), 3.91–4.00 (m, 3H, Glu-α and Gly-α,α') and 4.56 (dd, J = 7.7 and 5.0 Hz, 1H, Cys-α); MS m/z 407 $(MH)^+$; MS/MS collisional activation of m/z 407 afforded the product ion spectrum depicted in Figure 1.

Synthesis of SBuC. When the same procedure used for the synthesis of SBuG was followed with cysteine in place of GSH, SBuC was obtained in 35% yield: ^{1}H NMR δ 0.86 (t, J = 7.4 Hz, 3H, $NC_3H_6CH_3$), 1.21–1.39 (m, 2H, $NC_2H_4CH_2CH_3$), 1.40–1.50 (m, 2H, $NCH_2CH_2C_2H_5$), 3.15–3.35 (m, 3H, Cys-β and $NCH_2C_3H_7$), 3.52 (dd, J = 15.4 and 3.7 Hz, 1H, Cysβ'), and 4.08 (dd, J = 6.5, 3.7 Hz, 1H, Cys-α); MS m/z 221 $(MH)^+$; MS/MS collisional activation of m/z 221 afforded product ions at m/z 204 $(M + H - NH_3)^+$, 122 (cysteine + H)$^+$, 105 $(122 - NH_3)^+$, 100 $(C_4H_9NHCO)^+$, 87 $(105 - H_2O)^+$, 76 (glycine + H)$^+$, and 57 $(C_4H_9)^+$.

Synthesis of SPrG. This conjugate was prepared in 65% yield by the reaction of GSH with propyl isocyanate, according to the procedure employed to synthesize SBuG: ^{1}H NMR δ 0.84 (t, J = 7.4 Hz, 3H, $NC_2H_4CH_3$), 1.40–1.51 (m, 2H, $NCH_2CH_2CH_3$), 2.10–2.22 (m, 2H, Glu-β,β'), 2.48 (t, J = 7.6 Hz, 2H, Glu-γ,γ'), 3.10–3.24 (m, 3H, Cys-β and $NCH_2C_2H_5$), 3.40 (dd, J = 15.0 and 5.0 Hz, 1H, Cys-β'), 3.69–3.85 (m, 3H, Glu-α and Gly-α,α'), and 4.60 (dd, J = 7.7 and 5.0 Hz, 1H, Cys-α); MS m/z 393 $(MH)^+$; MS/MS collisional activation of m/z 393 afforded product ions at m/z 318 (M + H − glycine)$^+$, 264 (M + H − pyroglutamate)$^+$, 179 (cysteinylglycine + H)$^+$, 162 $(179 - NH_3)^+$, 161 ($C_3H_7NHCOSCH_2CH{=}NH_2^+$), 130 (pyroglutamate + H)$^+$, and 76 (glycine + H)$^+$.

Identification and Quantification of SBuG in Rat Bile. Four male Sprague-Dawley rats (200–300 g; Charles River Laboratories, Wilmington, MA) were anesthetized with urethane (1.4 g kg^{-1}, ip). The bile duct of each rat was isolated and cannulated with PE-10 tubing. Benomyl (100 mg kg^{-1}; Dalvi, 1992) was given by ip injection as a suspension in 0.5% methyl cellulose (25 mg mL^{-1}), and bile was collected for 4.5 h. A control experiment was conducted in parallel in which vehicle only was administered. Specimens of bile were filtered and analyzed by LC–MS/MS without further treatment. Quantification of SBuG in bile was accomplished by means of MS/MS with flow injection analysis. Thus, specimens of filtered bile (20 μL) were treated with SPrG (internal standard, 0.5 μg) and the mixture diluted to 1.0 mL with methanol/1% aqueous formic acid (1:1 v/v). Aliquots (20 μL) of this solution were introduced into the mass spectrometer by the flow injection technique, and the analyte and internal standard were detected by selected reaction monitoring (m/z 407 → 179 for SBuG, m/z 393 → 179 for SPrG). A standard curve was constructed similarly by adding a fixed amount (0.5 μg) of internal standard to specimens of control rat bile (20 μL) which had been treated with different quantities (0–2 μg) of SBuG. The resulting plot of peak area ratios for the signals for SBuG and SPrG against the concentration of SBuG, which was linear (r^2 = 0.999) over the concentration range of interest, was employed to determine the levels of SBuG in bile from benomyl-dosed rats.

Carbamoylation of Cysteine by SBuG *in Vitro*. Cysteine (1.9 mg, 16 μmol) was dissolved in prewarmed phosphate buffer (pH 7.4, 0.1 M, 4 mL) at 37 °C. The reaction was initiated by the addition of SBuG (1.3 mg, 3.2 μmol) and aliquots (100 μL) were withdrawn from the mixture at intervals over 320 min and acidified with TFA (1 μL). The samples were stored at −78 °C until analyzed by HPLC. *S*-(*N*-2-Chloroethylcarbamoyl)glutathione (SCG) (Davis *et al.*, 1993) was employed as an internal standard for HPLC analysis.

Carbamoylation of GSH by SBuC *in Vitro*. Glutathione (3.7 mg, 12 μmol) was dissolved in prewarmed phosphate buffer (pH 7.4, 0.1 M, 4 mL) at 37 °C. The reaction was initiated by adding SBuC (0.55 mg, 2.4 μmol) and both disappearance of substrate (SBuC) and

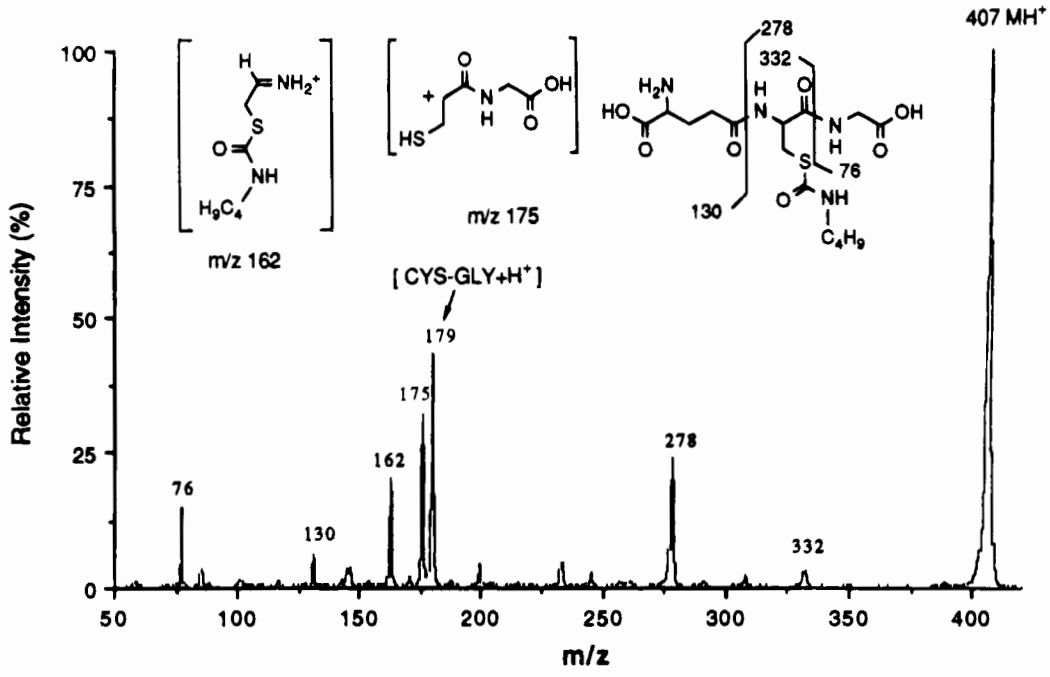


Figure 1. Product ion spectrum obtained by collision-induced dissociation of the MH^+ ion of synthetic SBuG (m/z 407). The origins of several characteristic product ions are indicated.

formation of product (SBuG) were followed by HPLC, as described above.

Cytotoxicity of Benomyl and SBuG toward Rat Hepatocytes *in Vitro*. A procedure described previously (Han *et al.*, 1990) was used with minor modifications. Briefly, hepatocytes were isolated according to standard procedures (Moldéus *et al.*, 1978) and incubations (1.5×10^6 cells mL^{-1}) were performed in Krebs buffer in silanized conical flasks which were rotated gently in a water bath held at 37 °C. Benomyl or SBuG was dissolved in Krebs buffer and added to the incubation media (final volume = 14 mL) to give a concentration of 0.2, 0.5, or 1.0 mM. Control incubations, which did not contain benomyl or SBuG, served to establish baseline levels of lactate dehydrogenase (LDH) at time zero (through disruption of cells with a microtip homogenizer, 20 kHz for 20 s) and of LDH release into the medium as a function of time (following brief centrifugation to remove intact hepatocytes). Incubations were gassed continuously with O_2/CO_2 (95:5) and triplicate aliquots (0.5 mL each) were removed at times 0, 2, 4, and 6 h for assay of LDH activity. This was performed spectrophotometrically by monitoring NADH formation at 340 nm, as described previously (Han *et al.*, 1990). The viability of each hepatocyte preparation was assessed at time zero and at the 6 h point by means of the trypan blue exclusion test.

RESULTS AND DISCUSSION

The present investigation, which was designed to assess whether benomyl serves as a precursor of BIC *in vivo*, was based on the results of recent studies which demonstrated that several alkyl isocyanates undergo metabolism via GSH conjugation. Since such conjugation is effectively reversible in nature under physiological conditions, the resulting GSH adducts may be viewed as latent forms of their reactive, toxic precursors *in vivo*. Following administration of benomyl to rats by ip injection, a targeted search was made for the GSH conjugate of BIC (SBuG) as a metabolite in bile. This was conducted with the aid of LC–ionspray MS/MS techniques, which have proven to be invaluable for the detection and quantitative analysis of labile GSH conjugates in specimens of crude bile. Selected reaction monitoring of the transition m/z 407 → 278, which corresponds to loss of the elements of pyroglutamic acid from the MH^+ ion of SBuG (Figure 1), was employed to detect the conjugate in bile from benomyl-treated rats (Figure 2A). Identification of the metabolite as SBuG was based on the fact that the corresponding authentic conjugate prepared by synthesis exhibited identical LC and MS/MS behavior to the benomyl metabolite (Figure 2B) and afforded a closely similar product ion spectrum upon collisional activation of the MH^+ ion (data not shown). Quantification of SBuG in bile, which was based upon selected reaction monitoring MS/MS (m/z 407 → 278 for SBuG and m/z 393 → 264 for the internal standard, SPrG), indicated that the fraction of the dose of benomyl excreted as this metabolite over 4.5 h was $0.84 \pm 0.24\%$ (mean ± SD, $N = 4$).

In view of the finding that *S*-(*N*-methylcarbamoyl)-glutathione (SMG) and *S*-(*N*-methylcarbamoyl)cysteine (SMC), the GSH and cysteine conjugates, respectively, of methyl isocyanate act as carbamoylating agents toward peptides and proteins (Pearson *et al.*, 1990), the reactivity of SBuG and SBuC toward the thiol groups of cysteine and GSH was examined *in vitro*. As depicted in Scheme 2, it was anticipated that SBuG would react with cysteine to yield SBuC and that SBuC would react similarly with GSH to form SBuG. The results of the former experiment are reproduced in Figure 3, which illustrates that SBuG donated the elements of BIC to cysteine in a time-dependent fashion. Closely similar results were obtained in the reaction of SBuC with GSH (data not shown). In both experiments, the disappearance of substrate followed apparent first-order kinetics, the rate constants for which were $(3.31 \pm 0.21) \times 10^{-3}$ min^{-1} for SBuG and $(7.3 \pm 1.1) \times 10^{-3}$ min^{-1} for SBuC. On the basis of these results, it may be concluded that SBuG and SBuC (and possibly other *S*-linked conjugates of BIC) serve as donors of the elements of BIC, and that

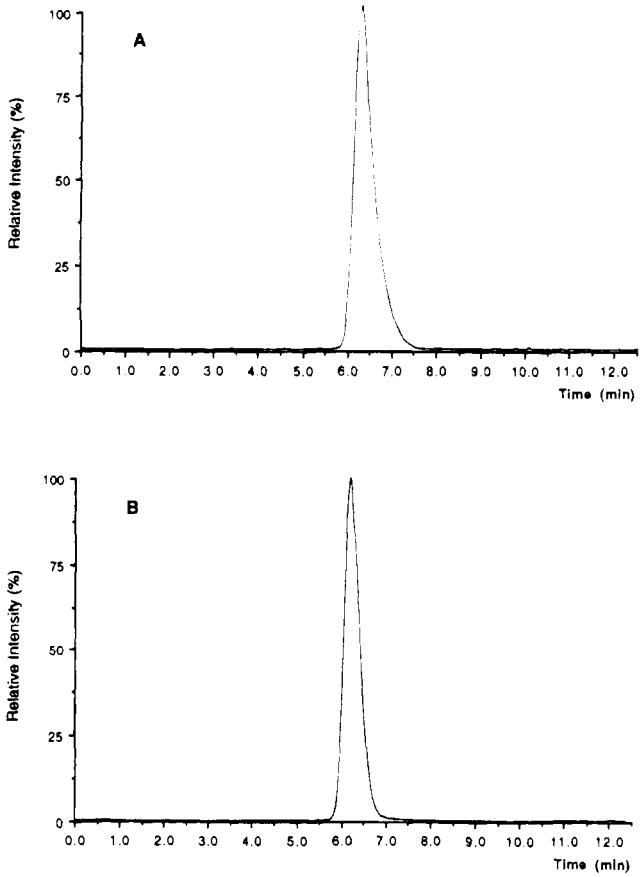


Figure 2. Ion current chromatograms obtained from LC–MS/MS analysis of a specimen of bile from a rat dosed with benomyl (100 mg kg^{-1} ip; chromatogram A) and of a synthetic standard of SBuG (chromatogram B). The analysis was performed on a Sciex API III triple quadrupole mass spectrometer, when SBuG was detected by selected reaction monitoring (SRM) of the transition m/z 407 → 278 (loss of the elements of pyroglutamic acid from the MH^+ ion).

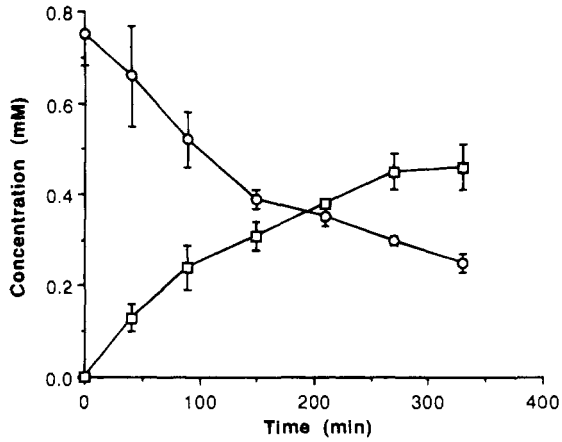


Figure 3. Carbamoylation of cysteine by SBuG (○) to form SBuC (□). SBuG (0.8 mM) was dissolved in phosphate buffer (0.1 M, pH 7.4) containing cysteine (4.0 mM) at 37 °C. SBuG and SBuC were quantified by HPLC using SCG as internal standard. Results are expressed as means ± SD (N = 3).

the cysteine adduct is a more reactive BIC donor than the corresponding GSH conjugate.

To determine the relative cytotoxic potential of SBuG and benomyl *in vitro*, freshly isolated rat hepatocytes were incubated with SBuG or benomyl at concentrations of 0.2, 0.5, and 1.0 mM and cell viability was assessed over a period of 6 h. As depicted in Figure 4, both compounds proved to be cytotoxic, in a time-dependent

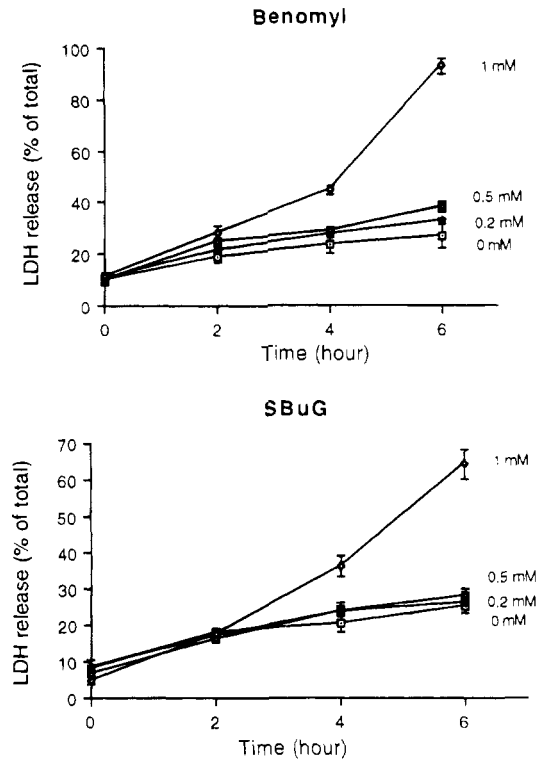


Figure 4. Cytotoxicity of benomyl (upper) and SBuG (lower) toward freshly isolated rat hepatocytes. Liver cells were incubated with test compounds (at 0.2, 0.5, and 1.0 mM) for 6 h, and cell killing was determined at 2-h intervals based on release into the medium of LDH. Results are expressed as means ± SD (N = 3).

manner, at the 1.0 mM level, benomyl being the more potent of the two agents. Under the conditions of these experiments, cell viability typically was in the order of 86 ± 4% at time zero and fell over 6 h to 58 ± 11% in control incubations. In contrast, when benomyl (1.0 mM) or SBuG (1.0 mM) was present, cell viability decreased to 12 ± 8% and 22 ± 12%, respectively, over 6 h. These results demonstrate that benomyl and SBuG both are cytotoxic to isolated rat liver cells *in vitro*, possibly as a result of their ability to release BIC at the cell membrane or in the intracellular space.

Traditionally, the reaction of electrophilic xenobiotics with GSH has been viewed as an important detoxification mechanism in both mammals and plants (Coles and Ketterer, 1990). However, it has become evident in recent years that certain classes of GSH conjugates may themselves be toxic (Koob and Dekant, 1991; Dekant and Vamvakas, 1993; Baillie and Kassahun, 1994). Adducts which are formed reversibly from the parent toxin fall into this category (van Bladeren, 1988), such as those derived from alkyl isothiocyanates (Bruggeman *et al.*, 1986) and alkyl isocyanates (Baillie and Kassahun, 1994). For example, SMG has been shown to be cytotoxic to isolated rat hepatocytes (Han *et al.*, 1990), to be embryotoxic to developing mouse embryos (Guest *et al.*, 1992), and to inhibit glutathione reductase *in vitro* (Jochheim and Baillie, 1994; Kassahun *et al.*, 1994). These effects have been attributed to the potent carbamoylating properties of the conjugate, which may be viewed as a "carrier" of the elements of MIC (Pearson *et al.*, 1990). It seems likely, therefore, that SBuG and related *S*-linked conjugates of BIC also will be toxic *in vivo* in view of their ability to act as latent forms of BIC. While the results of the present cytotoxicity experiments with isolated rat hepatocytes lend support to this

hypothesis, further studies will be needed to fully assess the role of BIC as a mediator of the adverse effects of benomyl *in vivo*.

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