Pergamon

Biochemical Pharmacology, Vol. 47, No. 7, pp. 1197–1206, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0105-G

SELECTIVE AND IRREVERSIBLE INHIBITION OF GLUTATHIONE REDUCTASE *IN VITRO* BY CARBAMATE THIOESTER CONJUGATES OF METHYL ISOCYANATE*

CLAUDIA M. JOCHHEIM and THOMAS A. BAILLIE†

Department of Medicinal Chemistry, School of Pharmacy, University of Washington, Seattle, WA 98195, U.S.A.

(*Received* 17 *June* 1993; *accepted* 26 *October* 1993)

Abstract—Exposure of yeast glutathione reductase (GR) *in vitro* to *S*-(*N*-methylcarbamoyl)glutathione (SMG) and *S*-(*N*-methylcarbamoyl)cysteine (SMC), two carbamoylating metabolites of methyl isocyanate (MIC), led to a time-dependent, irreversible loss of enzyme activity (50–90%) over a period of 3 hr. The extent of inhibition was dependent upon the concentration of these carbamate thioester conjugates (0.1 to 1.0 mM) and on the presence of NADPH (100 μM). Omission of NADPH markedly attenuated the inhibitory effects of both SMG and SMC, while oxidized glutathione (GSSG), the natural substrate of the enzyme, protected against the inhibition. Parallel experiments with the antineoplastic drug *N*,*N*′-bis-(2-chloroethyl)-*N*-nitrosourea (BCNU), a carbamoylating agent which is known to inhibit GR selectively, gave results that were similar to those obtained with the above conjugates. When analogs of SMG and SMC labeled with ^{14}C in the carbamoyl group were incubated with GR, radioactivity became bound covalently to the enzyme. These findings, together with the results of kinetic experiments on the release of GSH from SMG and cysteine from SMC, suggested that while both conjugates inhibit GR by carbamoylation of an active-site thiol(s), SMG exhibits a greater affinity for the active site than SMC. In contrast to the studies with GR, SMG and SMC failed to inhibit either glutathione-*S*-transferase (GST) or glutathione peroxidase (GPO) enzymes *in vitro*. It is concluded, therefore, that these conjugates most likely inhibit GR by carbamoylating free thiol groups in the active site of this enzyme, which are absent (or inaccessible) at the active-site of GST and GPO.

Key words: glutathione; glutathione reductase; carbamate thioesters; enzyme inhibition; carbamoylation

GR‡ is a flavoprotein that catalyzes the NADPH-dependent conversion of GSSG to the corresponding reduced form (GSH). This process takes place by way of the following two half-reactions, where "Enz" and "$EnzH_2$" denote oxidized (internal disulfide) and reduced (thiol) forms, respectively, of the enzyme [1]:

$$Enz + NADPH + H^+ \rightarrow EnzH_2 + NADP^+$$

$$EnzH_2 + GSSG \rightarrow Enz + 2GSH$$

GR plays a key role in maintaining cellular GSH homeostasis [2], and therefore represents a potential target for novel chemotherapeutic agents in the treatment of drug-resistant tumors. To date, the majority of studies on inhibition of GR have focused on BCNU (Fig. 1), a widely-used antitumor drug that has proven to be a potent and highly selective inhibitor of this enzyme both *in vitro* and *in vivo* [3–7]. BCNU decomposes spontaneously under physiological conditions to afford a number of products, one of which, CEIC, is believed to be responsible for the inhibition of GR following BCNU administration [4, 5]. Thus, it has been shown that CEIC carbamoylates a critical active site thiol in the reduced form of the enzyme and thereby inactivates the reductase [5]. Other nitrosoureas that collapse spontaneously to isocyanates probably inhibit GR in a similar fashion [4]. Interestingly, the labile *S*-linked GSH conjugate of CEIC, *S*-(*N*-[2-chloroethyl]-carbamoyl)glutathione (SCG), which has been identified recently as a biliary metabolite of BCNU in the rat, has been found to be equipotent with BCNU as an inhibitor of GR *in vitro* in rat hepatocytes [8]. This observation is consistent with the view that both BCNU and SCG serve as latent forms of CEIC.

The experimental antitumor agent NMF is known to undergo cytochrome P450-dependent metabolism, most likely via the reactive, electrophilic intermediate MIC, to yield the carbamate thioester conjugates SMG and SMC (Fig. 1) [9, 10]. These conjugates have been shown to be moderately unstable under physiological conditions and to act as donors of the elements of MIC to peptides and proteins,

* A preliminary account of this work was presented at the Society of Toxicology Annual Meeting, New Orleans, LA, March 15–19, 1993.

† Corresponding author: Dr. Thomas A. Baillie, Department of Medicinal Chemistry, School of Pharmacy, BG-20, University of Washington, Seattle, WA 98195, U.S.A. Tel. (206) 685-2468; FAX (206) 685-3252.

‡ Abbreviations: GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; BCNU, *N*,*N*′-bis-(2-chloroethyl)-*N*-nitrosourea; CEIC, 2-chloroethyl isocyanate; SCG, *S*-(*N*-[2-chloroethyl]carbamoyl)glutathione; NMF, *N*-methylformamide; MIC, methyl isocyanate; SMG, *S*-(*N*-methylcarbamoyl)glutathione; SMC, *S*-(*N*-methylcarbamoyl)cysteine; GST, glutathione-*S*-transferase; GPO, glutathione peroxidase; BSA, bovine serum albumin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); and CDNB, 1-chloro-2,4-dinitrobenzene.

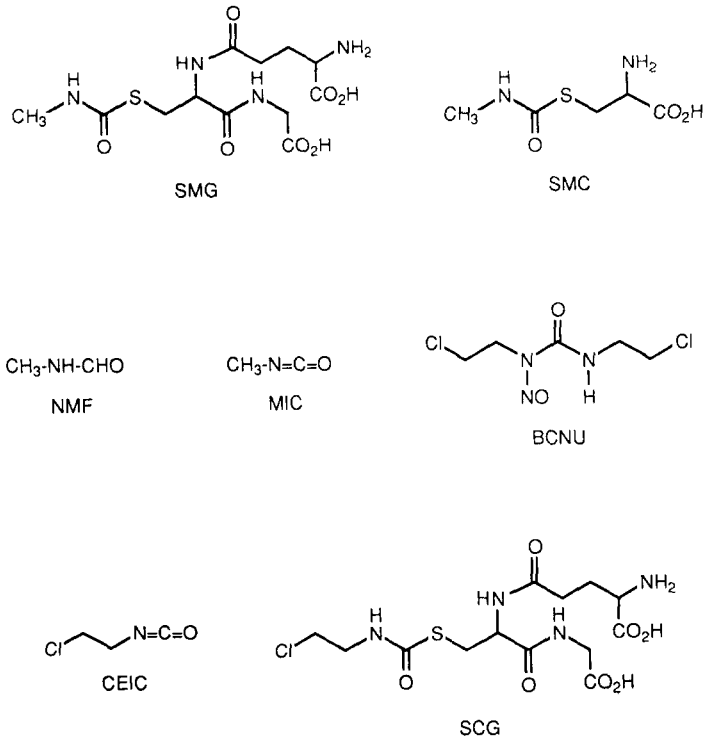

Fig. 1. Structures of compounds referred to in the text.

when free sulfhydryl groups are carbamoylated preferentially [11, 12]. By analogy with the behavior of CEIC and its metabolic precursors towards GR, therefore, it appeared possible that SMG and SMC also could serve as inhibitors of GR and thus contribute to the antitumor properties of NMF. In light of these considerations, the present *in vitro* study was designed to gain further information on the interaction of carbamate thioester conjugates with GR, and aimed to test the hypothesis that SMG and SMC carbamoylate, and thereby inhibit, GR. In view of the possibility that SMG, by virtue of its glutathionyl moiety, might serve as an active site directed inhibitor of the reductase, particular interest focused on determining whether SMG behaved differently from its cysteinyl analog, SMC. Moreover, information was sought on the role of NADPH on the inhibitory properties of these conjugates, since the reduced form of GR contains two free cysteinyl sulfhydryls at the active site which could serve as targets for carbamoylation, whereas in the oxidized form of the enzyme, these residues are linked via a relatively unreactive disulfide bridge. The form of GR obtained from yeast was adopted in these studies as a model for the corresponding human enzyme, since it has been shown that the sequence of the redox-active peptide located at the active site of both yeast and human erythrocyte GR is identical in all thirteen positions [13, 14].

MATERIALS AND METHODS

Materials

Bakers yeast GR (EC 1.6.4.2; purified by affinity chromatography), rat liver GST (EC 2.5.1.18, a mixture of isoforms) and bovine erythrocyte GPO (EC 1.11.1.9) were purchased from the Sigma Chemical Co. (St. Louis, MO) and used as received. Units of activity for these enzymes were reported by the manufacturer as follows: GR: 1.0 unit catalyzes the reduction of 1.0 μmol GS-SCoA/min at pH 7.4 and 37°; GST: 1.0 unit catalyzes the conversion of 1.0 μmol CDNB/min to its GSH conjugate at pH 6.5 and 25°; GPO: 1.0 unit catalyzes the H_2O_2-dependent oxidation of 1.0 μmol of GSH to GSSG/min at pH 7.0 and 25°.

SMG and SMC were obtained by synthesis, as described previously [15], and ^{14}C-labeled analogs of these conjugates were prepared as outlined below. BCNU was purchased from Bristol Laboratories (Evansville, IN), and GSH, GSSG, BSA, DTNB, NADPH and sodium azide were obtained from the Sigma Chemical Co. [1-^{14}C]Acetyl chloride (sp. act. 30 mCi/mmol) was purchased from ICN Biochemicals, Inc. (Costa Mesa, CA). Other chemicals were of reagent grade and were obtained from commercial sources.

Synthesis of [^{14}C-carbamoyl]*SMG*. A breakseal vial of [1-^{14}C]acetyl chloride (1 mCi) was partially immersed in a dry ice/acetone bath. The seal was broken and, after adding anhydrous toluene (1.0 mL), the contents were transferred to a cooled round-bottom flask containing unlabeled acetyl chloride (0.90 mL, 12.5 mmol) and sodium azide (3.0 g, 46 mmol). The vial then was rinsed with a further portion of toluene (6.0 mL), which was added to the contents of the round-bottom flask, and the

resulting mixture was heated slowly to reflux (90°). After a period of 2 hr at reflux, the flask was allowed to cool to ambient temperature (30 min) and the reaction product was isolated by microdistillation (34° at atmospheric pressure) into a receiving vessel that was held in an acetone/dry ice bath. This afforded a toluene solution (1.5 mL) of radiolabeled MIC (CH_3-N=^{14}C=O), the yield of which (4.1 mmol; 33%) was determined from the ratio of the methyl resonances in the ^{1}H-NMR spectrum of the distillate which were derived from toluene (2.45 ppm) and MIC (3.05 ppm). A portion of this solution (0.75 mL, containing 2.05 mmol [^{14}C]MIC) was transferred to a second round-bottom flask containing a solution of GSH (307 mg, 1.0 mmol) in acetonitrile/water (7:3, v/v; 6.0 mL). The reaction mixture was stirred at ambient temperature for 2 hr, following which the solvent was removed under reduced pressure. The residue was subjected to semi-preparative HPLC (C_{18} column; 250 mm × 10 mm i.d.; 5 μm particle size; mobile phase: 10 mM potassium phosphate buffer, pH 4.3, flow rate 3.0 mL/min) which yielded [^{14}C]SMG (160 mg, 0.439 mmol; 44%). This material was judged to be 97% chemically pure and 100% radiochemically pure by HPLC analysis, and exhibited a specific activity of 25 μCi/mmol.

Synthesis of [^{14}C-carbamoyl]*SMC.* The labeled conjugate was obtained in a fashion similar to that described above for [^{14}C]SMG. Thus, reaction of cysteine (121 mg, 1.0 mmol) with [^{14}C]MIC (2.05 mmol) in aqueous acetonitrile afforded the desired conjugate (yield: 114 mg, 0.64 mmol; 64%). The [^{14}C]SMC obtained by this procedure was judged to be 99% chemically pure and 100% radiochemically pure by HPLC analysis and exhibited a specific activity of 25 μCi/mmol. This material was used directly without purification.

Methods

Instrumentation. Proton NMR spectra (300 MHz) were recorded on a Varian VXR 300 spectrometer (Varian Associates, Palo Alto, CA). Samples were dissolved in ^2H_2O and chemical shifts are expressed as parts per million (δ) relative to residual H_2O (δ 4.67). Spectrophotometric assays were carried out using a Hewlett–Packard model 8451A diode array instrument. HPLC was performed using a Beckman model 112 dual pump instrument equipped either with a Hewlett–Packard model 1040A diode array detector or a Radiomatic model A-100 radioactivity flow detector. Radioactivity covalently bound to protein was measured by liquid scintillation counting in a Packard model 2000 CA TriCarb instrument.

Concentration-dependent inhibition of GR by SMG. Incubations of GR (0.075 nmol, 0.016 U) with SMG (0, 125, 333, 800 μM or 3.0 mM) contained BSA (1.0 mg) and NADPH (100 μM) in 100 mM phosphate buffer (pH 7.4; total volume = 1.0 mL). Mixtures were preincubated at 20° for 10 min, following which the reaction was initiated by adding GSSG (0–175 μM), and enzyme activity was determined as described below.

Time-dependent inhibition of GR by SMG, SMC and BCNU. In these experiments, the inhibitor concentration was held constant at 500 μM and the preincubation time was varied from 10 min to 3 hr. Incubation mixtures contained GR (0.016 U), BSA (1.0 mg) and NADPH (100 μM), and the reaction was initiated by adding GSSG (to a concentration of 500 μM). The activity of GR was determined as described below.

Effect of NADPH on inhibition of GR. These studies were carried out in a manner similar to the time-dependent experiments, except that the enzyme was preincubated for 3 hr either in the absence of NADPH or in the presence of this cofactor (100 μM).

Effect of GSSG on inhibition of GR. Incubations contained GR (0.17 nmol, 3.5 U), SMG or SMC (500 μM) and NADPH (100 μM) in 100 mM phosphate buffer (pH 7.4; total volume = 1.0 mL), and were performed in the presence or absence of GSSG (250 μM). After a preincubation period of 1.5 hr at 20°, aliquots of the incubation medium (20 μL) were added to 100 mM phosphate buffer (0.78 mL), and reaction was initiated by the addition of GSSG (500 μM). The activity of GR then was determined as described below.

Test for irreversible inhibition. Incubations were performed as described in the above experiment with the exception that the substrate, GSSG, was omitted. Preincubations were carried out for 1.5 and 3 hr. Following each incubation, the product was subjected to gel chromatography (6-mL Sephadex G-25 size-exclusion column eluted with 100 mM potassium phosphate buffer; pH 7.4), and fractions (1.0 mL) were collected and assayed for enzyme activity, protein concentration and presence of inhibitor. By this means, it was found that the majority of the GR eluted in fraction 3 where it was well-separated from both SMG and SMC (which eluted in fractions 5–7 and 10–11, respectively). Therefore, fraction 3 was taken for assay of GR activity. Protein concentration was measured by the micro method of Bradford [16] (using purified GR to generate the standard curve), whereas reaction with bicinchoninic acid reagent [17] also detected the cysteine in SMG and SMC [18] and was used to establish the overall profile of compounds in the column effluent.

Measurement of GR activity. This was determined by an assay method based on the consumption of NADPH which accompanies the reduction of GSSG. The loss of NADPH was quantified spectrophotometrically by measuring the decrease in absorbance of the incubation mixture at 340 nm over a 2-min period [19].

Measurement of GST activity. This was based on the method of Habig *et al.* [20]. Incubation mixtures contained GST (0.029 U), EDTA (1.0 mM), GSH (500 μM) and inhibitor (500 μM) in 100 mM phosphate buffer (pH 7.4; total volume = 1.0 mL). Following a 3-hr preincubation period, these mixtures were treated with CDNB (to 1.0 mM), and the formation of the corresponding GSH conjugate was followed spectrophotometrically at 340 nm for 2 min.

Measurement of GPO activity. This was based on the method of Paglia and Valentine [21]. Incubation mixtures contained GPO (0.1 U), EDTA (1.0 mM), GSH (500 μM), NADPH (300 μM) and inhibitor (500 μM) in 100 mM phosphate buffer (pH 7.4; total volume = 1.0 mL). Following a 3-hr preincubation

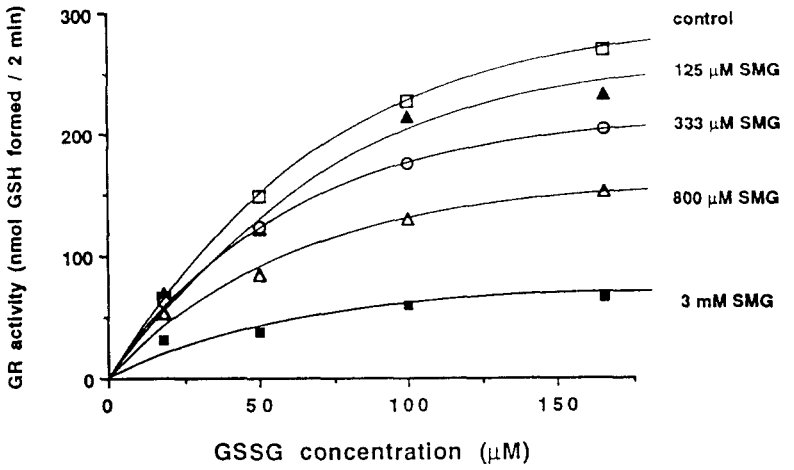

Fig. 2. Concentration-dependent inhibition of GR by SMG. The enzyme was preincubated for 10 min with varying concentrations of SMG (0 to 3.0 mM) and a fixed concentration of NADPH (100 μM), following which GSSG (0–175 μM) was added to initiate the reaction. Enzyme activity was measured as described under Materials and Methods.

period, these mixtures were treated with GR (1.0 U) and cumene hydroperoxide (to 40 μM), and the consumption of NADPH was followed spectrophotometrically at 340 nm for 2 min.

In all of the above assays, each sample was run in parallel with its own control (no inhibitor added), and the results are expressed as per cent of control.

Covalent binding of SMG and SMC to GR. GR (5.82 nmol) was incubated for 3 hr with [^{14}C]SMG or [^{14}C]SMG (5.82 μmol; 25 μCi/mmol) and NADPH (5.0 μmol) in 500 mM potassium phosphate buffer (pH 7.4; 0.5 mL). The protein was precipitated by the addition of trichloroacetic acid (TCA; 3.0 M; 0.1 mL) and separated from non-covalently bound inhibitor by repeated 2-mL washes with 1% TCA over a nylon filter (0.45 μm). When no further radioactivity was detected in the filtrates (typically after 16 washes), the covalently bound radioactivity was determined by measuring the ^{14}C in the filter-bound protein by liquid scintillation counting.

Decomposition of SMG and SMC in aqueous medium. The time-dependent release of GSH from SMG (500 μM) or cysteine from SMC (500 μM) was measured spectrophotometrically using the method of Ellman [22]. Incubations were carried out in 100 mM potassium phosphate buffer (pH 7.4) at 37° for 40 min either in the presence of catalytic amounts of GR (0.016 U) or in the absence of enzyme. To minimize auto-oxidation of liberated thiols, the buffer was degassed prior to each incubation. At intervals during the incubation period, aliquots (1.0 mL) of the test solutions were added to a 3 mM solution of DTNB in 100 mM (pH 7.4) phosphate buffer (50 μL), and the absorbance at 412 nm was measured. The concentrations of GSH and cysteine then were calculated by reference to appropriate standard curves for these thiols.

Initial reaction of SMG and SMC with GR ("burst" experiment). A sample containing SMG or SMC (450 μM) was allowed to react with GR (8.3 μM; 948 U) and NADPH (200 μM) in degassed phosphate buffer (100 mM; pH 7.4). At 1, 4, 10 and 30 min, aliquots (1.0 mL) of the reaction mixture were passed through C_{18} cartridges (Bakerbond, J. T. Baker) to remove the (thiol-containing) enzyme. The cartridges were washed with 1.0 mL of the above buffer, and the combined eluents (obtained by applying Ar pressure to the cartridges) were taken for assay of thiol content. This was performed by reacting portions (1.0 mL) of the eluent with DTNB solution (50 μL), as described above.

Statistics. Statistical analyses of the data were performed by Student's *t*-test. A probability value of $P < 0.05$ was considered to denote a statistically significant difference. Data are presented as mean values ± SD.

RESULTS

When GSSG was incubated with GR in the presence of a fixed concentration of NADPH (100 μM), the disulfide was reduced to GSH in a process that exhibited Michaelis–Menten kinetics, characterized by K_m and V_{max} values of 110 μM and 3.0 μmol GSH formed/min/nmol enzyme, respectively. As illustrated in Fig. 2, preincubation of GR for 10 min with the GSH conjugate SMG (0 to 3.0 mM) led to a concentration-dependent inhibition of enzyme activity with substrate (GSSG) concentrations in the range of 0–175 μM. In these experiments, an SMG concentration of 800 μM decreased the V_{max} of the reaction to approximately 50% of control values for each concentration of GSSG examined. The inhibition also proved to be time-dependent, as shown in Fig. 3 which depicts the fall in GR activity resulting from preincubation of the enzyme with a fixed concentration of SMG (500 μM). Parallel time-course experiments were conducted with the cysteine conjugate SMC (500 μM) and also with BCNU (500 μM), which was included

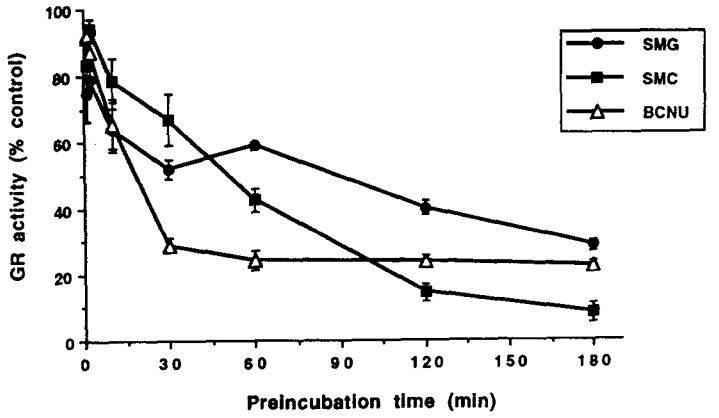

Fig. 3. Time-dependent inhibition of GR by SMG, SMC and BCNU. The enzyme was preincubated for varying periods of time (10–180 min) with a fixed concentration of both inhibitor (500 μM) and NADPH (100 μM), following which GSSG (500 μM) was added to initiate the reaction. Enzyme activity was measured as described under Materials and Methods. Results are expressed in terms of corresponding control activities (no inhibitor added) and represent means ± SD (N = 3). Control activity at time zero was 135 ± 4.4 nmol GSH formed/min and, following a 3-hr preincubation in the absence of inhibitor, it was 90 ± 5.7 nmol GSH formed/min.

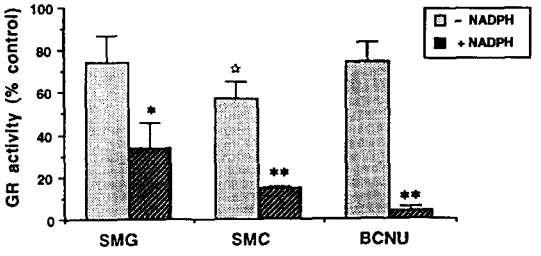

Fig. 4. NADPH-dependent inhibition of GR by SMG, SMC and BCNU. The enzyme was preincubated for 180 min with a fixed concentration of inhibitor (500 μM), either in the presence of NADPH (100 μM) or in the absence of this cofactor. Following the addition of GSSG (500 μM) to initiate the reaction, enzyme activity was measured as described under Materials and Methods. Results are expressed in terms of corresponding control activities (no inhibitor added) and represent means ± SD (N = 3). The mean control activity was 87.4 ± 6.6 nmol GSH formed/min. Key: (*) significantly different ($P < 0.05$) from the corresponding "minus NADPH" value, (**) significantly different ($P < 0.01$) from the corresponding "minus NADPH" value, and (☆) significantly different ($P < 0.05$) from the corresponding control value.

as a positive control (Fig. 3). In all cases, GR activity fell rapidly over the first 30 min, SMG being a more potent inhibitor than SMC over this time interval. Beyond the 30-min point, however, there were differences in the behavior of the three compounds in that prolonged preincubation of the enzyme with SMG and BCNU led to only a slight additional decrease in activity, whereas preparations treated with SMC continued to lose activity throughout the duration of the 3-hr experiment. Indeed, at the 3-hr time point, SMC was the most potent of the inhibitors studied, having decreased GR activity to some 10% of control values.

For all three compounds, inhibition of GR was dependent upon the redox state of the enzyme since inclusion of NADPH (100 μM) in preincubation mixtures increased markedly the inhibition measured at 3 hr (Fig. 4). However, SMC, which was a potent inhibitor at this time point, reduced GR activity to 60% of the control value even in the absence of NADPH (Fig. 4).

To examine whether the thioester conjugates themselves were responsible for inhibition of the enzyme, as opposed to free MIC generated by spontaneous breakdown of SMG and SMC in aqueous medium, a study was conducted to examine the stability of these carbamate thioesters in aqueous pH 7.4 buffer at 37°. In view of the high chemical reactivity of MIC, which precludes its direct measurement in aqueous solutions, breakdown of the conjugates of interest was assessed by quantifying the release of free GSH (or cysteine). This was examined in the absence and presence of catalytic amounts of GR (0.016 U; 0.075 nmol). As shown in Fig. 5, both SMG and SMC decomposed slowly in aqueous buffer in a process that was not accelerated by the presence of GR. Calculation of the initial rates of decomposition (using data collected over the first 8 min) indicated that SMC produced free cysteine *ca.* 4 times more rapidly than SMG gave rise to GSH. Unimolecular rate constants for the release of GSH from SMG in the absence and presence of GR were $12.6 \times 10^{-6}\,min^{-1}$ and $13.5 \times 10^{-6}\,min^{-1}$, respectively, while the corresponding values for the release of cysteine from SMC were $50.9 \times 10^{-6}\,min^{-1}$ and $53.1 \times 10^{-6}\,min^{-1}$. Since these rate constants are relatively low, it may be concluded that free MIC, generated by spontaneous decomposition of SMG and SMC in aqueous medium, does not play a significant role in

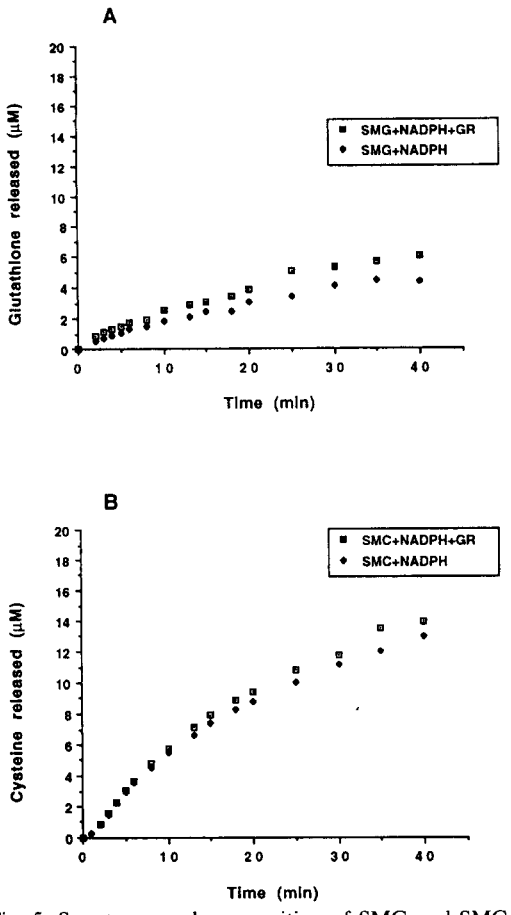

Fig. 5. Spontaneous decomposition of SMG and SMC in aqueous buffer (pH 7.4, 37°) in the absence and presence of GR (0.016 U). The release of GSH from SMG (500 μM) (A) or of cysteine from SMC (500 μM) (B) was measured spectrophotometrically as described under Materials and Methods.

the inhibition of GR by the parent carbamate thioesters.

When GR was incubated in the presence of the radiolabeled inhibitors [^{14}C]SMG and [^{14}C]SMC, radioactivity became bound irreversibly to the enzyme, consistent with the known carbamoylating properties of these *S*-linked conjugates (Fig. 6). In the presence of NADPH, the stoichiometry of the binding was 1.3 nmol SMG bound per nmol GR, and 2.9 nmol SMC bound per nmol GR. These values should be considered as minimum levels of covalent modification, however, because it was noted that the process of denaturing and repeatedly washing the enzyme led to gradual removal of radioactivity which may have been bound covalently, but was present in the form of a chemically labile linkage (see below). In the absence of NADPH, the binding of ^{14}C from both inhibitors was decreased significantly; in fact, only SMC gave rise to measurable binding to the enzyme when the cofactor was omitted.

To determine whether the inhibition of GR was irreversible in nature, enzyme that had been exposed to SMG or SMC (500 μM) in the presence of NADPH was subjected to gel chromatography to remove any non-covalently bound inhibitor. Residual GR activity in appropriate column fractions was found to correspond to *ca.* 85% of control values following preincubation with SMG for 1.5 hr, and *ca* 55% after 3 hr. Similar values were obtained for the enzyme that had been preincubated with SMC. Thus, enzyme activities following gel chromatography were only slightly higher than those measured prior to the chromatographic step (Fig. 3), demonstrating that the inhibition was largely irreversible.

In an experiment designed to assess the influence of the natural substrate, GSSG (250 μM), on the inhibition of GR by SMG (500 μM) over 1.5 hr, it was found that essentially no loss of enzyme activity occurred when GSSG was present. Thus, GR activity was 98.0 ± 0.5% (N = 3) of the control (non-inhibited) value in the presence of GSSG, whereas in the absence of the disulfide the enzyme activity

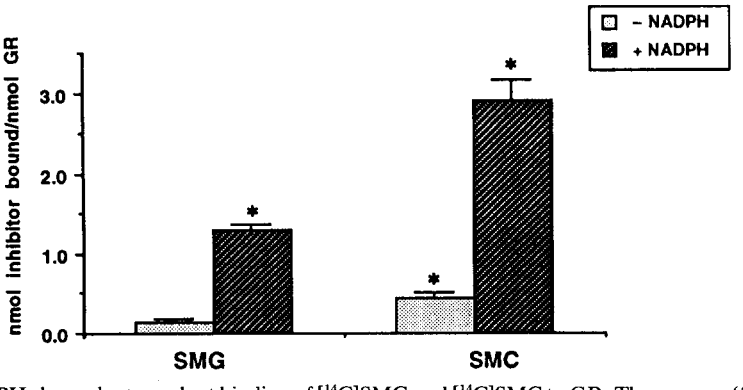

Fig. 6. NADPH-dependent covalent binding of [^{14}C]SMG and [^{14}C]SMC to GR. The enzyme (5.82 nmol) was incubated for 180 min with a fixed concentration of each inhibitor (5.82 μmol), either in the presence of NADPH (5.0 μmol) or in the absence of this cofactor. Then covalent binding of ^{14}C to the enzyme protein was determined as described under Materials and Methods. Values are means ± SD (N = 4). Key: (*) significantly different ($P < 0.001$) from background radioactivity.

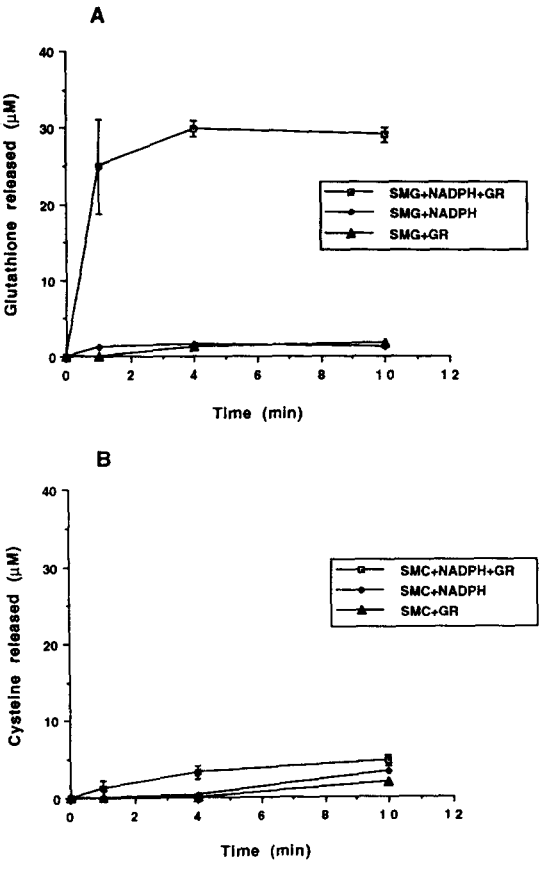

Fig. 7. Initial reaction of SMG and SMC with GR ("burst" experiment). SMG (450 μM) (A) and SMC (450 μM) (B) were allowed to react with GR (8.3 μM; 948 U) and NADPH (200 μM) at pH 7.4 and 37°. At intervals over 10 min, aliquots of incubation medium were removed, the enzyme was separated, and soluble free thiols were determined as outlined in Materials and Methods. Values are means ± SD (N = 3).

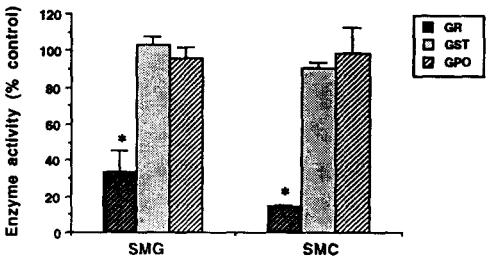

Fig. 8. Effects of SMG and SMC on the activities of GSH-dependent enzymes. GR, GST and GPO were preincubated for 180 min with a fixed concentration (500 μM) of SMG or SMC, following which enzyme activities were determined as outlined under Materials and Methods. Results are expressed in terms of corresponding control activities (no inhibitor added) and represent means ± SD (N = 3). Control activities for the three enzymes after a 180-min preincubation were as follows: GR: 78.1 ± 3.9 nmol GSH formed/min; GST: 21.6 ± 3.2 nmol GS-dinitrobenzene formed/min; GPO: 98.4 ± 11.1 nmol GSSG formed/min. Key: (*) significantly different ($P < 0.01$) from the corresponding control values.

fell to 72.0 ± 7.8% of control. The corresponding values for parallel experiments with SMC were 98.0 ± 1.4% in the presence of GSSG and 68.0 ± 1.3% in its absence. This substrate-dependent protection against inhibition suggests that both SMG and SMC exert their inhibitory effects on GR through interactions at the active site of the enzyme.

In an attempt to investigate further the mechanism by which SMG and SMC inhibit GR, a "burst" experiment was carried out with each conjugate, similar in design to kinetic experiments employed to titrate the active sites of enzymes with their respective substrates. In the general case where a substrate ("S") is converted by an enzyme ("Enz") to two products, "P_1" and "P_2" (where P_1 and P_2 may, or may not, be identical), the reaction can be represented as follows:

$$\text{Enz} + \text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Enz}\cdot\text{S} \xrightarrow[\text{P}_1]{k_1'} \text{Enz-Int} \xrightarrow{k_2} \text{Enz} + \text{P}_2$$

where "Enz·S" represents the initial non-covalent complex formed between enzyme and substrate and k_1, k_{-1}, $k_{1'}$, and k_2 denote the rate constants of the individual steps. When the substrate reacts rapidly with the enzyme to release P_1, after which "Enz-Int" breaks down slowly to P_2 with regeneration of the native enzyme, then the molar concentration of P_1 released during the initial burst "$[P_1]_\pi$" is related to the effective molar concentration of the enzyme "$[E]_o$" by the following expression:

$$[P_1]_\pi = [E]_o\left(\frac{k_1'}{k_1' + k_2}\right)^2 \quad [23].$$

However, in the case where "S" is an active site-directed irreversible inhibitor of the enzyme, initial formation of a reversible enzyme–inhibitor complex is followed by *covalent* modification of the enzyme. In this situation, k_2 becomes negligible and the above expression simplifies to $[P_1]_\pi = [E]_o$. Thus, by determining $[P_1]_\pi$ experimentally, it is possible to calculate $[E]_o$ which, in turn, reveals the stoichiometry of the covalent binding of the substrate to the enzyme.

Applying the above considerations to the interaction of SMG and SMC with GR, it was reasoned that if either conjugate served as an active-site directed inhibitor, then a "burst" of P_1 (GSH or cysteine, respectively) should be observed when the parent carbamate thioester was added to the active enzyme. Moreover, the stoichiometry of $[P_1]_\pi$ relative to $[E]_o$ should provide a measure of the number of sites on the enzyme which become carbamoylated.

The results of this "burst" experiment are shown in Fig. 7. When SMG (450 μM) was incubated with GR (8.3 μM) in the presence of the cofactor NADPH (200 μM), a fast burst of GSH was observed which attained a maximum concentration of 30 μM after 4 min (Fig. 7A). This inferred a 4:1 stoichiometry

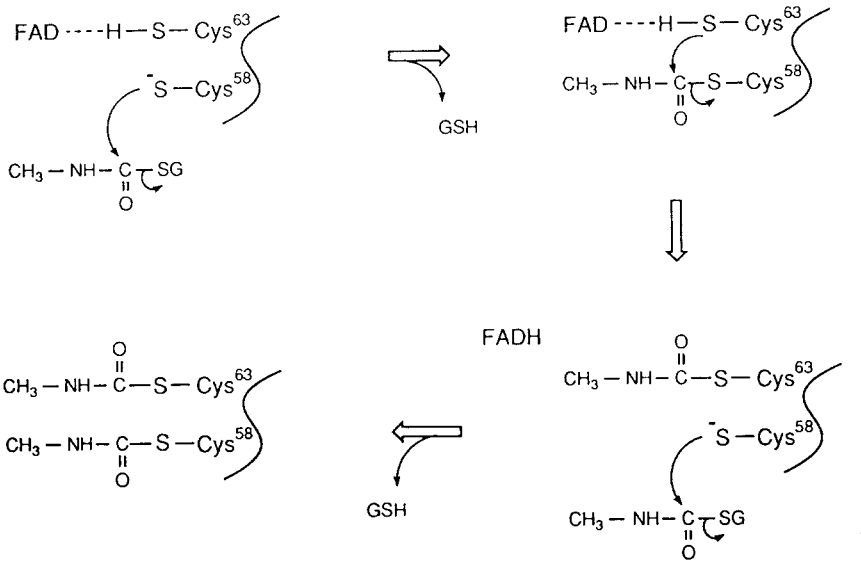

Fig. 9. Proposed mechanism for the irreversible inactivation of GR by SMG. For details, see text.

of modified residues per mole of GR. The corresponding control incubation performed in the absence of NADPH gave rise to only small amounts of GSH which were similar to those observed in studies of the spontaneous decomposition of SMG in aqueous buffer (Fig. 5A). In contrast to the study with SMG, the "burst" experiment with SMC (Fig. 7B) produced only a small amount of cysteine (3 μM at 4 min), suggesting that the value for k^{-1} for SMC is much larger (or the value for k_1' is much lower) than that for SMG. Prolonged incubation of GR with SMC afforded a similar amount of cysteine as the control incubations where NADPH or GR was omitted. These results suggest that although SMC appears to inhibit GR by modifying the active site of the enzyme, the mechanism by which it acts must differ in some respects from that of the GSH conjugate.

Finally, in an experiment to assess the selectivity of SMG and SMC as inhibitors of the GSH-dependent enzymes, GR, GST and GPO, incubations with each enzyme were performed at a fixed concentration of inhibitor (500 μM), and residual activity was determined at the 3-hr time point. The results, which are presented in Fig. 8, indicate that of the three enzymes of interest, only GR was susceptible to inhibition by SMG and SMC.

DISCUSSION

The present study demonstrated that the carbamate thioester conjugates SMG and SMC inhibit yeast GR *in vitro* in a time- and concentration-dependent manner. The inhibition was enhanced in the presence of NADPH (the required cofactor for GR activity), and occurred at a rate that was much faster than that of the spontaneous decomposition of these conjugates to MIC. The latter observation indicates that SMG and SMC *per se*, rather than free MIC, are the active inhibitory species. Also, it was found that inhibition of GR was accompanied by carbamoylation of the enzyme, was essentially irreversible in nature, and was blocked by the presence of GSSG, the natural substrate.

In considering possible mechanisms for the inhibitory effects of SMG and SMC on the reductase, it is instructive to review the accepted catalytic cycle of the enzyme. As discussed above, the overall process by which GSSG is converted to GSH may be viewed in terms of two half-reactions, the first of which (reduction of the enzyme by NADPH) is believed to be rate-limiting. In this step, a hydride is transferred from NADPH to the prosthetic group (FAD) which, in turn, donates two reducing equivalents to an internal disulfide (Cys_{58}-Cys_{63} in the human enzyme) at the active site. In the second half-reaction, the thiolate anion (Cys_{58}-S^-) that is formed by this reductive cleavage acts as a nucleophile towards the substrate, GSSG, forming the mixed disulfide Cys_{58}S-SG and releasing one molecule of GSH. The second sulfhydryl moiety generated in the reductive half-reaction (Cys_{63}SH), which is believed to be stabilized by a charge-transfer interaction with the isoalloxazine ring of FAD, completes the catalytic cycle by attacking the neighbouring mixed disulfide to release a second molecule of GSH and regenerate the native (oxidized) form of GR. Based on this sequence of events, and given that carbamate thioester conjugates such as SMG and SMC exhibit a preference for carbamoylation of thiol groups on proteins [11], it appears that the thiolate anion of Cys_{58} represents a likely target for the initial covalent modification of GR by SMG (Fig. 9). In fact, BCNU has been shown to inhibit human erythrocyte GR by carbamoylating the enzyme at this specific residue [5]. As proposed in Fig. 9, nucleophilic attack by the sulfhydryl of Cys_{63} on the carbamoylated Cys_{58}

would result in intramolecular transfer of the methylcarbamoyl moiety to Cys_{63} and regeneration of the Cys_{58} thiolate anion for further reaction. A second carbamoylation cycle then would lead to the fully modified active site in which thiol residues are carbamoylated. However, since GR is a homodimer with two functional active sites, it would be expected that a total of four methylcarbamoyl moieties would be incorporated per mole of enzyme, and this prediction is in good agreement with the results of the "burst" experiment with SMG (Fig. 7A).

The scenario outlined above may reasonably be viewed as mechanism-based inhibition, since carbamoylation of GR by SMG is dependent upon the normal catalytic activity of the enzyme. Protection by GSSG against the inhibitory properties of carbamate thioesters also is consistent with this view, and it is interesting to note that, under physiological conditions, molecules of GSH liberated in the reaction of SMG with GR will undergo auto-oxidation to GSSG which, in turn, will block further inhibition of the enzyme. Thus, the effects of SMG on GR should be self-limiting, whereas the corresponding cysteine conjugate, SMC, would not be expected to behave in this manner. Indeed, the present studies revealed several differences between SMC and SMG in terms of the nature of their interaction with GR, notably in the "burst" experiment when SMC did not release free cysteine rapidly into the medium (Fig. 7B). The most plausible explanation for these differences is that while SMG is directed specifically to the active site of GR by virtue of its glutathionyl moiety, SMC is not similarly directed (although clearly it does bind to the active site). In support of this hypothesis, a variety of non-carbamoylating GSH conjugates have been shown to bind reversibly to the active site of GR [24]. This mechanism therefore accounts for the fact that the time–course of inhibition by SMC, which normally is a more effective donor of the elements of MIC than the corresponding GSH conjugate [25], is slower than that observed with SMG.

Lastly, it was shown that of the three GSH-dependent enzymes that play a key role in protecting cells against electrophilic xenobiotics and reactive oxygen species, namely GR, GST and GPO, only GR was inactivated by SMG and SMC. Thus, the carbamate thioesters of interest, together with the nitrosourea BCNU which liberates CEIC spontaneously in aqueous medium, appear to inactivate GR with a high degree of selectivity which may reflect the absence of critical, sterically accessible, sulfhydryl groups at the active sites of the other enzymes. Hence, free isocyanates, metabolic precursors of isocyanates [9, 26], carbamoylating nitrosoureas [4] and carbamate thioester conjugates [8, 9, 27, 28], which gain access to the active site of GR, would be predicted to serve as irreversible inhibitors of this enzyme.

Acknowledgements—The authors would like to thank Dr. William M. Atkins (Department of Medicinal Chemistry, University of Washington) for valuable discussions during the course of this work. This research was supported by a fellowship (to C.M.J.) from the Dr. Mildred Scheel Foundation for Cancer Research and by a Research Grant from the National Institutes of Health (ES05500), which are gratefully acknowledged.

REFERENCES

1. Sweet WL and Blanchard JS, Human erythrocyte glutathione reductase: Chemical mechanism and structure of the transition state for hydride transfer. *Biochemistry* **30**: 8702–8709, 1991.
2. Ackerboom TPM, Bilzer M and Sies H, The relationship of biliary glutathione disulfide efflux and intracellular glutathione disulfide content in perfused rat liver. *J Biol Chem* **257**: 4248–4252, 1982.
3. Brundrett RB, Cowens JW, Colvin M and Jardine I, Chemistry of nitrosoureas. Decomposition of deuterated 1,3-bis(2-chloroethyl)-1-nitrosourea. *J Med Chem* **19**: 958–961, 1976.
4. Babson JR and Reed DJ, Inactivation of glutathione reductase by 2-chloroethyl nitrosourea-derived isocyanates. *Biochem Biophys Res Commun* **83**: 754–762, 1978.
5. Karplus PA, Krauth-Siegel RL, Schirmer RH and Schulz GE, Inhibition of human glutathione reductase by the nitrosourea drugs 1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea. A crystallographic analysis. *Eur J Biochem* **171**: 193–198, 1988.
6. Frischer H and Ahmad T, Severe generalized glutathione reductase deficiency after antitumor chemotherapy with BCNU [1,3-bis(chloroethyl)-1-nitrosourea]. *J Lab Clin Med* **89**: 1080–1091, 1977.
7. Cohen MB and Duvel DL, Characterization of the inhibition of glutathione reductase and the recovery of enzyme activity in exponentially growing murine leukemia (L1210) cells treated with 1,3-bis(2-chloroethyl)-1-nitrosourea. *Biochem Pharmacol* **37**: 3317–3320, 1988.
8. Davis MR, Kassahun K, Jochheim CM, Brandt KM and Baillie TA, Glutathione and *N*-acetylcysteine conjugates of 2-chloroethyl isocyanate. Identification as metabolites of *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea in the rat and inhibitory properties toward glutathione reductase *in vitro*. *Chem Res Toxicol* **6**: 376–383, 1993.
9. Threadgill MD, Axworthy DB, Baillie TA, Farmer PB, Farrow KC, Gescher A, Kestell P, Pearson PG and Shaw AJ, Metabolism of *N*-methylformamide in mice: Primary kinetic deuterium isotope effect and identification of *S*-(*N*-methylcarbamoyl)glutathione as a metabolite. *J Pharmacol Exp Ther* **242**: 312–319, 1987.
10. Slatter JG, Rashed MS, Pearson PG, Han D-H and Baillie TA, Biotransformation of methyl isocyanate in the rat. Evidence for glutathione conjugation as a major pathway of metabolism and implications for isocyanate-mediated toxicities. *Chem Res Toxicol* **4**: 157–161, 1991.
11. Pearson PG, Slatter JG, Rashed MS, Han D-H and Baillie TA, Carbamoylation of peptides and proteins *in vitro* by *S*-(*N*-methylcarbamoyl)glutathione and *S*-(*N*-methylcarbamoyl)cysteine, two electrophilic *S*-linked conjugates of methyl isocyanate. *Chem Res Toxicol* **4**: 436–444, 1991.
12. Baillie TA and Slatter JG, Glutathione: A vehicle for the transport of chemically reactive metabolites *in vivo*. *Acc Chem Res* **24**: 264–270, 1991.
13. Worthington DJ and Rosemeyer MA, Glutathione reductase from human erythrocytes; Molecular weight, subunit composition and aggregation properties. *Eur J Biochem* **60**: 459–466, 1975.
14. Krohne-Ehrich G, Schirmer RH and Untucht-Grau R, Glutathione reductase from human erythrocytes;

Isolation of the enzyme and sequence analysis of the redox-active peptide. *Eur J Biochem* **80**: 65–71, 1971.
15. Han D-H, Pearson PG, Baillie TA, Dayal R, Tsang LH and Gescher A, Chemical synthesis and cytotoxic properties of *N*-alkylcarbamic acid thioesters, metabolites of hepatotoxic formamides. *Chem Res Toxicol* **3**: 118–124, 1990.
16. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
17. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC, Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**: 76–85, 1985.
18. Wiechelman KJ, Braun RD and Fitzpatrick JD, Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal Biochem* **175**: 231–237, 1988.
19. Colman RF, Glutathione reductase (yeast). *Methods Enzymol* **17** (Part B): 500–503, 1971.
20. Habig WH, Pabst MJ and Jakoby WB, Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**: 7130–7139, 1974.
21. Paglia DE and Valentine WN, Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* **70**: 158–169, 1967.
22. Ellman G, Tissue sulfhydryl groups. *Arch Biochem Biophys* **82**: 70–77, 1959.
23. Fersht A, The absolute concentration of enzymes. In: *Enzyme Structure and Mechanism*, pp. 143–146. W. H. Freeman & Co., New York, 1985.
24. Sies H, Intracellular effects of glutathione conjugates and their transport from the cell. In: *Glutathione Conjugation, Mechanisms and Biological Significance* (Eds. Sies H and Ketterer B), pp. 175–192. Academic Press, New York, 1988.
25. Pearson PG, Slatter JG, Rashed M, Han D-H, Grillo MP and Baillie TA, *S*-(*N*-Methylcarbamoyl)glutathione: A reactive *S*-linked metabolite of methyl isocyanate. *Biochem Biophys Res Commun* **166**: 245–250, 1990.
26. Slatter JG, Davis MR, Han D-H, Pearson PG and Baillie TA, Studies on the metabolic fate of caracemide, an experimental antitumor agent, in the rat. Evidence for the release of methyl isocyanate *in vivo*. *Chem Res Toxicol* **6**: 335–340, 1993.
27. Jayaram HN, Lui MS, Plowman J, Pillwein K, Reardon MA, Elliott WL and Weber G, Oncolytic activity and mechanism of action of a novel L-cysteine derivative, L-cysteine, ethyl ester, *S*-(*N*-methylcarbamate) monohydrochloride. *Cancer Chemother Pharmacol* **26**: 88–92, 1990.
28. Németh L, Somfai-Relle S, Kellner B, Sugár J, Bognár R, Farkas J, Bálint J, Pályi I, Tóth K, Szentirmay Z, Somosy Z and Pokorny E, Study of the antitumoral activity of *S*-carbamoyl-L-cysteine derivatives in animal experiments. *Arzneimittelforschung* **28**: 1119–1123, 1978.