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Characterization of melphalan-glutathione adducts whose formation is catalyzed by glutathione transferases

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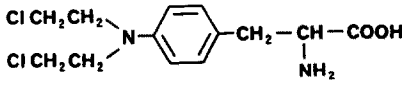
A number of authors have described an association between elevated sulfhydryl levels and resistance to alkylating agents. Current explanations for the development of resistance to antineoplastic nitrogen mustards, such as melphalan, include evidence of impaired drug intake [1] and increased levels of intracellular glutathione [2–4]. Recently, Vistica and coworkers [5] reported that murine L1210 cells resistant to melphalan contain elevated levels of both reduced glutathione and glutathione-*S*-transferase. However, none of these studies has suggested the direct formation of a melphalan-glutathione adduct(s) as being a potentially important consideration in this phenomenon. The objective of this study was to characterize the conjugation products between melphalan (**1**) and glutathione whose formation is catalyzed by both cytosolic and microsomal glutathione-*S*-transferases. The reaction products have been identified as the diglutathione conjugate formed by displacing both chloride groups in the mustard moiety, and a monoglutathione conjugate formed by displacing one chloride group.



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Materials and methods

Melphalan (L-phenylalanine mustard, L-PAM) was supplied by the Division of Cancer Treatment, NCI, (Bethesda, MD). Reduced glutathione (GSH) was obtained from the Sigma Chemical Co. (St. Louis, MO). [^{14}C]Melphalan (sp. act. 10 mCi/mmole) was obtained from the Drug Development Branch of the National Cancer Institute. The radiolabeled compound was purified by high pressure liquid chromatography (HPLC) using a C_{18} reversed-phase column (Bondapak, Waters Associates, Milford, MA) and a methanol-1% aqueous acetic acid gradient as the mobile phase. The purified drug was stored as a solution in 100 mM HCl at −70°.

Immobilized microsomal glutathione-*S*-transferases were prepared from rabbit and human livers by a modification of the method of Lehman *et al.* [6] and Pallante *et al.* [7]. Male New Zealand white rabbits (5–8 lb) were obtained from Bunnyville and given a 0.05% solution of phenobarbital *ad lib.* for 2 weeks. Food was withheld 24 hr prior to killing the animals with CO_2; hepatic microsomal protein was immobilized immediately. Histologically normal human liver was obtained from surgical resection samples, frozen immediately on dry ice, and stored at −80° before immobilization. Specific activity of the bound glutathione-*S*-transferase was determined spectrophotometrically using 1-chloro-2,4-dinithrobenzene as the assay standard [8]. Activity in nmoles/min/mg protein was: rabbit, 171; human, 108.

Incubations with immobilized microsomal glutathione transferases. The incubation mixture contained: melphalan (1.0 mM, 10.3 mg), GSH (3.0 mM, 27.6 mg), and packed immobilized Sepharose beads from rabbit or human liver (28 ml) in aqueous phosphate buffer (0.1 M, pH 7.4). The reaction was run at 37° for 1 hr, followed by filtration. The aqueous filtrate was evaporated to a volume of 1–2 ml and analyzed by thin-layer chromatography (TLC). A control reaction was carried out in the absence of immobilized enzyme. Products of the incubation were purified by HPLC using the method described below. Purified conjugates

were characterized by TLC and positive fast atom bombardment (FAB) mass spectrometry.

Incubations with cytosolic glutathione transferases. The incubation mixture contained [^{14}C]melphalan (50 μM, 10 μCi/μmole); glutathione or 2-mercaptoethanol (1 mM); and potassium phosphate buffer, pH 7.4, in mouse liver 105,000 × g supernatant fraction (50 μl, 3.4 mg protein/ml). Incubations were carried out for 30 min at 37°, at which time the reaction was stopped by the addition of an equal volume of 10% trichloroacetic acid (TCA). Following centrifugation, an aliquot (50 μl) of the acid-soluble supernatant fraction was separated by high pressure liquid chromatography using a microBondapak C_{18} column (Waters) eluted with a methanol gradient (5–50% methanol) in 1% acetic acid. Fractions (1 ml) were analyzed by liquid scintillation counting.

Base-catalyzed hydrolysis of melphalan. Melphalan (6.56 mmoles, 2.0 mg) was dissolved in 1.0 ml NaOH (0.1 M) and allowed to react at room temperature for 3 hr. The solution was neutralized with 0.1 M HCl. The mono- and dihydroxy products of L-PAM were purified by HPLC using the method described below. Purified products were characterized after trimethylsilylation by electron impact (EI) mass spectrometry.

HPLC purification methods. Melphalan-GSH conjugates were separated using a Beckman model 344 HPLC system with a Kratos Spectra-flow model 757 variable wavelength detector (254 nm), a C_{18} Altex Ultrasphere ODS column (5 μm; 150 × 4.6 mm), a gradient mobile phase (0.1 M ammonium acetate: methanol, 10 to 100% in 25 min) at a flow rate of 1.0 ml/min. Mono- and dihydroxy derivatives of melphalan were separated on the same column, using a gradient mobile phase of aqueous acetic acid (1%): aqueous acetic acid (1%)–acetonitrile (50:50), 10 to 100% in 30 min. Eluents were collected, evaporated to dryness under vacuum, and stored at −20°.

Thin-layer chromatography. Silica gel plates (5 × 10 cm) with u.v. fluorescent indicator (E. Merck, Darmstadt, West Germany) were used with a mobile phase of absolute ethanol–aqueous ammonia (70:30). Products were visualized by both u.v. and ninhydrin spray reagent (0.2% in ethanol).

Mass spectrometry. Fast atom bombardment mass spectra were measured on a Kratos MS 50 mass spectrometer with a Kratos FAB source and DS-55 data System (accelerating voltage 8 kV and resolution 3000). Samples were dissolved in 25 μl methanol or acetonitrile containing 1% HCl (1 M). A 10-fold excess of thioglycerol was used as the liquid matrix. Samples were bombarded with a xenon atom beam at 7–8 kV accelerating voltage. Spectra were plotted as averages of 10–15 scans, 1000 to 200 daltons at 30 sec/decade.

Electron impact spectra were measured on a Kratos MS 80 mass spectrometer using 70 eV electrons, 4 kV accelerating voltage, 225° source temperature and 1000 resolution. Samples were dissolved in pyridine (25 μl) and derivatized at 25° for 30 min with bis-(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (75 μl) from the Pierce Chemical Co., Rockford, IL. Samples were introduced by direct probe. The mass range 600 to 200 daltons was scanned at 3 sec/decade.

Results and discussion

Incubation of melphalan with glutathione and immobilized enzyme from rabbit liver resulted in the formation of a complex mixture which was separated by high pressure liquid chromatography as shown in Fig. 1. Upon TLC analysis, product peaks A through F reacted with ninhydrin. Mass spectrometric analysis of products C and E provided molecular weights and analyses of chlorine content consistent with structures **2** and **3**. Both TLC and HPLC analysis of the control reaction indicated that these glutathione conjugates were formed less than 1% as abundantly in the absence of immobilized protein. Incubation of melphalan with microsomal protein solubilized and immobilized from human liver provided product mixtures which contained the same three glutathione conjugates as the rabbit transferase product mixture, judged by TLC and HPLC retention times.

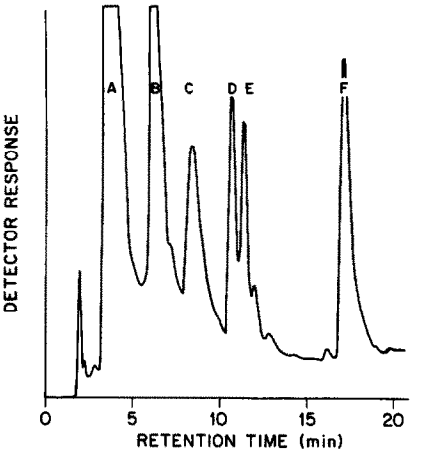
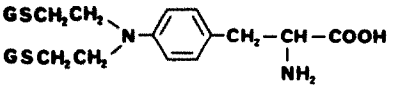


Fig. 1. High pressure liquid chromatogram of the product mixture from the incubation of melphalan, glutathione and immobilized solubilized rabbit liver microsomal protein. In the text, arguments are made to identify peak A as dihydroxy melphalan, B as monohydroxy melphalan, C as diglutathionyl melphalan, E as monoglutathionyl melphalan, and F as melphalan.



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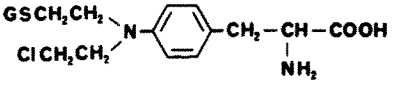

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Fast atom bombardment mass spectrometry has been successfully employed for analysis of intact glutathione conjugates [7] and was used in this study. A partial FAB mass spectrum of product C, the diglutathionyl adduct, is shown in Fig. 2. A peak corresponding to the protonated molecular ion of the intact conjugate was observed at m/z 847. Sodium and potassium adduct peaks were seen at m/z 869 and m/z 885, respectively, commonly encountered in FAB spectra. This compound is of particular interest, since very few diglutathionyl conjugates have been reported and characterized [9, 10].

Product E was characterized by its FAB spectrum as the monochloromonoglutathionyl adduct, with protonated molecular ions of mass 576 and 578, and chlorine-containing fragment ions of mass 254 and 256 (Fig. 3) formed by β-cleavage. The mass spectrum of peak D suggests that it is a glutathione conjugate of phenylalanine. This structure and the mechanism of its formation are still under investigation. Peak F in the chromatogram (Fig. 1) was found to correspond in retention time to authentic melphalan and peaks A and B to hydrolysis products of melphalan in which two and one chloride groups had been displaced by hydroxyl groups.

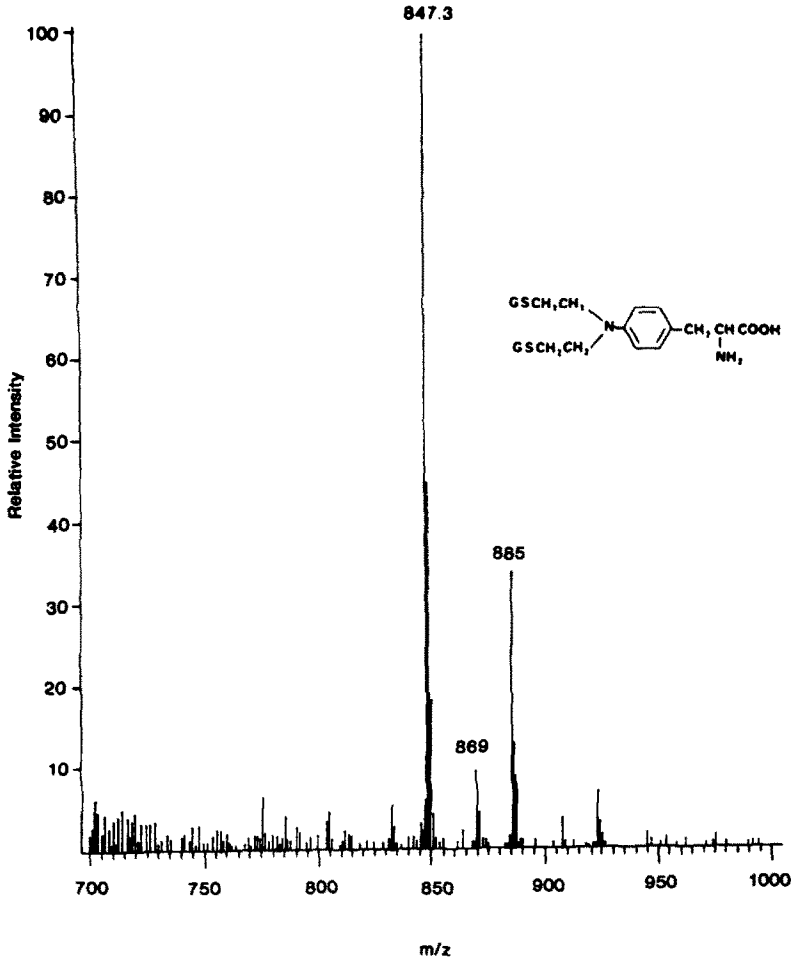


Fig. 2. Partial positive ion fast atom bombardment mass spectrum of the diglutathione conjugate, peak C in Fig. 1.

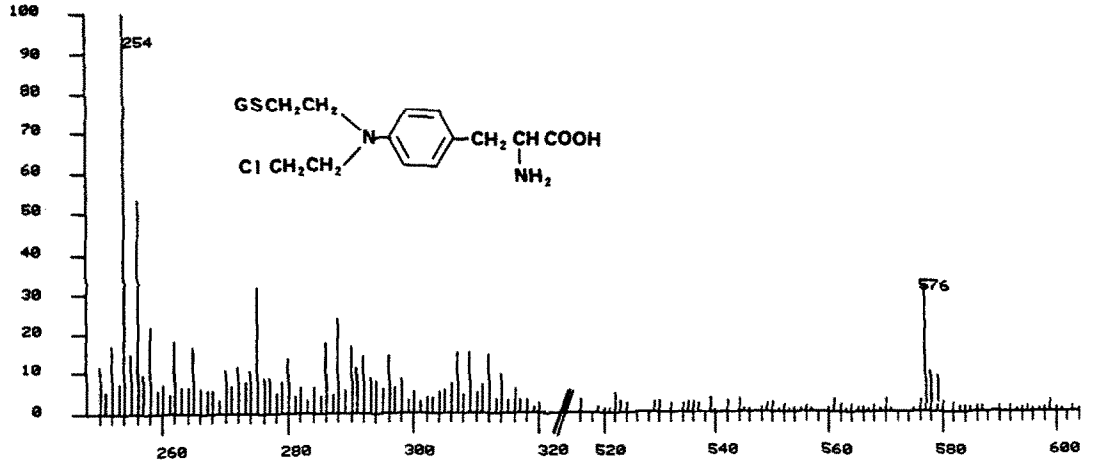
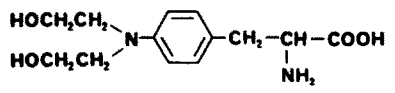


Fig. 3. Partial positive ion fast atom bombardment mass spectrum of the monoglutathione conjugate, peak E in Fig. 1.

Although mono- and dihydroxy melphalan derivatives have been reported as metabolites [11, 12], heretofore proof of their structures has been inferential. These compounds were prepared in aqueous base and purified by reversed-phase HPLC. Acetonitrile was employed in the mobile phase to avoid esterification by methanol. Trimethylsilylation of the purified products followed by electron impact mass spectrometric analysis (Figs. 4 and 5) identified the most polar component of the mixture as dihydroxy melphalan (**4**) and the next component eluted as the monohydroxy-derivative (**5**). These co-eluted with peaks A and B in Fig. 1. Derivatized dihydroxy melphalan gave a molecular radical cation of mass 556 and an M-15 ion of mass 541 (Fig. 4). The base peak at m/z 338 may reflect multiple cleavage in the mustard group [13]. The monohydroxy derivative produced molecular radical cations of masses 502 and 504, reflecting the presence of a chlorine atom (Fig. 5). The base peak at m/z 308 may correspond to a fragment formed by loss of the mustard portion of the molecule. The fragment of mass 385 presumably arose from loss of trimethylsilylated COOH. Neither hydroxylated melphalan product provided definitive spectra when subjected to FAB mass spectrometry in several matrices.



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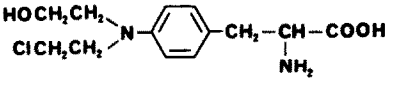

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Radioisotope-labeled melphalan was also incubated with cytosolic protein from mouse liver cells. The product mixtures from those incubations were characterized by HPLC as monohydroxy-, dihydroxy-, and glutathione-conjugated melphalan, accounting, respectively, for 4, 10 and 21% of the total radioactivity. In a control incubation without added glutathione, the presence of endogenous glutathione probably accounts for 5% of the label being incorporated into the glutathione conjugate fraction. When protein was omitted but glutathione added, the conjugate fraction carried 8% of the label. When 2-mercaptoethanol was substituted for glutathione, with and without protein, 0 and 7% of the label was incorporated into glutathione conjugates.

The characterization of conjugates between melphalan and glutathione whose formation is catalyzed by both cytosolic and microsomal glutathione-*S*-transferase and particularly by enzymes from human liver should stimulate further studies of metabolism and of the development of

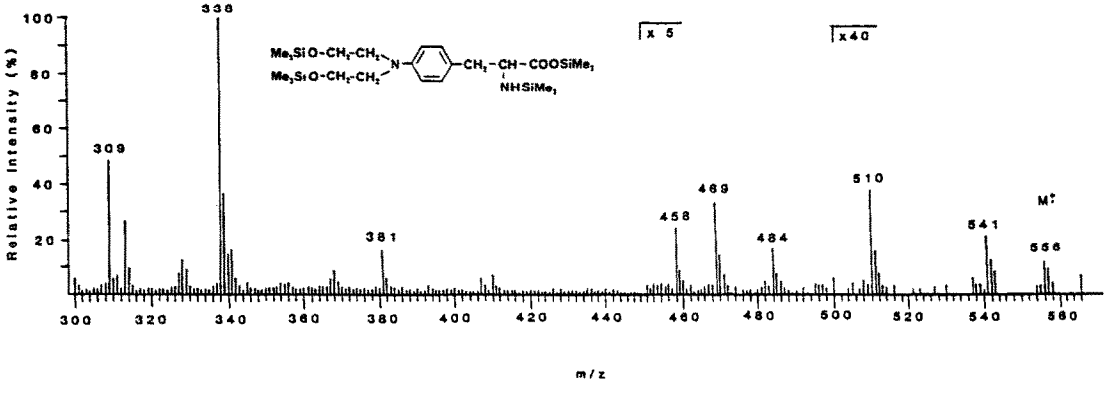

Fig. 4. Partial electron impact mass spectrum of the trimethylsilylated hydrolysis product, peak A in Fig. 1. This partial spectrum is normalized to the most intense peak above m/z 300.

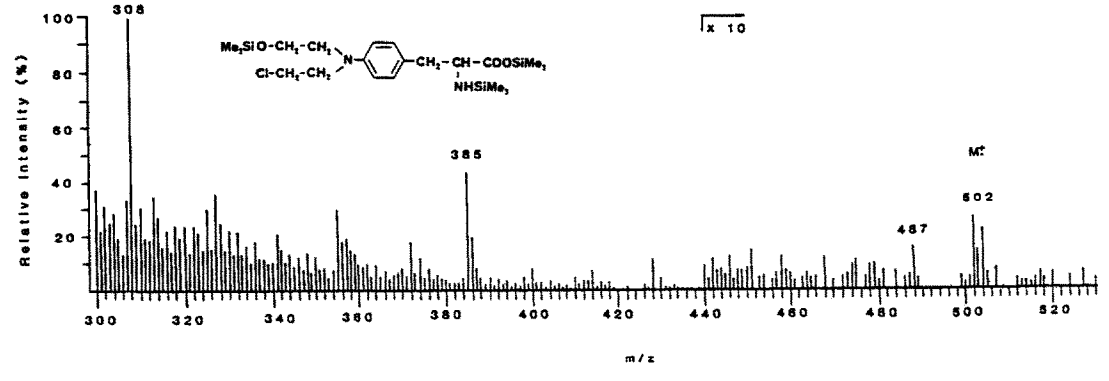

Fig. 5. Partial electron impact mass spectrum of the trimethylsilylated hydrolysis product, peak B in Fig. 1. This partial spectrum is normalized to the most intense peak above m/z 300.

resistance for this drug. While the alkylating properties of these adducts have not yet been investigated, it would be anticipated that these conjugates are less reactive than melphalan itself. If so, conjugation with glutathione could provide a mechanistic basis for the development of cellular resistance to the drug, already known to be associated with elevated glutathione and transferase levels. In addition, development of the analytical methodology to characterize these adducts may be useful in further study of the resistance phenomenon *in vivo*. Currently, studies are under way to determine whether glutathione conjugates of melphalan are formed in melphalan-resistant cells.

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Glutathione turnover in perfused rabbit lung. Effect of external glutathione

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In the past few years there has been an interest in studying the utilization of external glutathione (GSH), GSH esters, or its precursors for augmenting the intracellular reserve of GSH [1–9]. In the body, the kidney is the primary organ which utilizes circulating plasma GSH by virtue of the high transpeptidase activity [10, 11]. Utilization of external GSH by perfused rat lung for conjugation reaction and as cellular GSH reserve has also been reported [8, 9]. These authors suggest the involvement of transpeptidase activity in the catabolism of the circulating GSH leading to the utilization of precursors thus formed, by the lungs. Since there are considerable species differences between rat and rabbit lung drug-metabolizing enzymes [12, 13], we wished to study the pulmonary utilization of externally provided GSH in rabbit lungs.

Materials and methods

Male New Zealand white rabbits (2 to 2.5 kg), obtained from a local source and maintained at our Central Animal Facilities, were used for the present study. Rabbits were treated with diethylmaleate (DEM) (6 mmoles/kg in 3 ml corn oil/kg, i.p.), and the controls received the corn oil vehicle only, 1 hr prior to surgery. Rabbits were anesthetized (Nembutal, 50 mg/kg) and heparinized (sodium heparin, 1000 units/rabbit) by i.v. injection through the marginal ear vein. Lungs were surgically removed from normal and DEM-pretreated rabbits. The oxygenated Krebs–Ringer bicarbonate buffer, supplemented with 4.5% bovine serum albumin (BSA) and 5 mM glucose (pH 7.4), was used for perfusion. For some perfusions, BSA was replaced by dextran (5%). The perfusion apparatus and the entire procedure for the recirculating system have been described previously [14, 15]. Lung perfusions were allowed to equilibrate for 10 min before the addition of GSH. Perfusate samples were collected over 1 ml perchloric acid (PCA) (final concentration 1.75%) at different time intervals. Upon termination of the perfusion after 1 hr, lungs were quickly washed with 75 ml saline in order to wash out any perfusate from the vasculature. Tissue samples were minced in PCA (at a final concentration of 1.75%), homogenized, and centrifuged. GSH and oxidized glutathione (GSSG) in the tissue and perfusate were analyzed by the HPLC method of Reed *et al.* [16].

DEM-pretreated lungs were found to deteriorate to a greater extent and more rapidly during perfusion. This could have occurred due to a rapid and severe loss of cellular GSH. Histological changes due to O_2 sensitivity in DEM-treated rat lungs have been reported recently [17]. Since depletion of GSH was a necessary experimental manipulation for some of our experiments, the criterion of 10% increase in lung weight was relaxed to 20%. Lower doses of DEM (1 and 3 mmoles/kg) failed to deplete GSH