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Research Article

Intrahepatic Delivery of Glutathione by Conjugation to Dextran

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Glutathione was covalently attached to dextran (T-40) by the CNBr activation method. The compound obtained was a water-soluble powder containing 10 (w/w%) glutathione, which was gradually released from the conjugate in aqueous media. Mice depleted of glutathione by treatment with buthionine sulfoximine, a potent inhibitor of γ-glutamylcysteine synthetase, exhibited a significant increase in hepatic glutathione level after intravenous injection of the conjugate. In mice given a lethal dose of acetaminophen, the survival rate increased progressively with coadministration of the conjugate, whereas little improvement was found when free glutathione was given. The conjugate maintained the serum transaminase activities at lower level after acetaminophen administration. These findings suggest that the dextran conjugate of glutathione is transported into hepatic cells and is intracellulary hydrolyzed to free form, which protects mice from hepatotoxicity due to acetaminophen.

KEY WORDS: glutathione; dextran conjugate; intrahepatic delivery; buthionine sulfoximine; acetaminophen; hepatotoxicity.

INTRODUCTION

Glutathione (GSH) is a tripeptide (L-γ-glutamyl-L-cysteinylglycine) which is the most ubiquitous intracellular non-protein thiol. It plays an important role in the protection of cells against damage by toxic substances, reactive oxygen compounds, and radiation (1). Since the detoxication potential of the liver is intimately connected to the GSH status of the organ (2,3), it has been widely used in the treatment of hepatic disorders, allergies, poisonings of xenobiotics and heavy metals, etc. However, extracellularly administered GSH is impermeable to the hepatic cells and has a short half-life in the circulation due to its rapid renal degradation (4–6).

Covalent attachment of proteins to soluble dextrans can greatly increase the half life of the protein in blood circulation (7–10). Further, Melton *et al.* (11,12), working with carboxypeptidase G_2 conjugated to soluble dextrans, found a pronounced uptake of both CNBr-activated dextran and dextran-enzyme conjugate by the liver. The present work was stimulated by the hypothesis that a dextran conjugate of GSH might be more stable in the systemic circulation and more readily transported into hepatic cells than GSH itself. In this paper we describe the preparation of a GSH-dextran conjugate, the properties of the conjugate, and the hepatic GSH levels in mice treated with it. Protection of mice pretreated with the conjugate against acetaminophen-induced hepatotoxicity was also examined.

MATERIALS AND METHODS

Chemicals and Reagents. GSH and oxidized glutathione (GSSG) were obtained from Sigma Chemical Co., St. Louis, Mo. Dextran (T-40, MW 43,900, M_n = 26,200) was purchased from Pharmacia Fine Chemicals Co., Sweden. All other chemicals and reagents were of the highest grades commercially available.

Preparation of a Dextran Conjugate of GSH. GSH was covalently attached to dextran by the cyanogen bromide activation method (13). To a stirred solution of dextran (0.2 g) in water (20 ml) cyanogen bromide was added in three portions (40, 40, and 30 mg). The pH was maintained at 11.0 during this process by adding 4 *M* NaOH. Six minutes after the final addition of cyanogen bromide, the pH was adjusted to 6.5 by the addition of 0.1 *M* HCl. Then GSH (0.4 g) was added maintaining the pH at 6.5 and the coupling reaction was allowed to proceed overnight at 4°C. The reaction mixture was washed repeatedly with 0.1 *M* CH_3COOH by pressure dialysis using a Toyo UHP-43 ultrafiltration cell incorporating a UP-20 membrane (Toyo, Tokyo) and, finally, concentrated to a volume of 10 ml. Excess reagents and low molecular by-products of the reaction were removed by this process. The product, a dextran conjugate of GSH(D-GSH), was obtained from lyophilization of the final solution.

Estimation of the Degree of Binding of GSH to Dextran by Measuring the Sulfhydryl Group. Sulfhydryl group of D-GSH was determined by the method of Ellman (14) as follows. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB; 3.96 mg)

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was dissolved in 1 ml of phosphate buffer (pH 7.0, $\mu = 0.1$). The DTNB reagent (0.02 ml) was added to 3.0 ml of the sample of GSH or D-GSH in 0.1 *M* phosphate buffer (pH 8.0). The absorbance of the color developed was measured at 412 nm. The molar absorptivity of GSH was 13,990 $M^{-1}\,cm^{-1}$.

Detection of Dextran. Dextran was measured by the phenol-sulfuric acid method (15), calibrated against glucose. As Marshall and Rabinowitz pointed out (16), cyanogen bromide treatment of dextran resulted in a marked decrease in the color production in the phenol-sulfuric acid reaction. Therefore, sugar contents of D-GSH were estimated as apparent values.

Determination of GSH by HPLC. The amount of GSH released from the conjugate *in vitro* was determined by HPLC. Chromatography was carried out using a Shimadzu liquid chromatographic system (LC-6A) with a variable-wavelength UV detector (SPD-6A) operated at 200 nm (Shimadzu, Kyoto, Japan). A 150 × 4.6-mm, 5-μm-particle size, C_{18} reversed-phase column (Cosmosil $5C_{18}$, Nakarai Chemical Co., Kyoto, Japan) was used at ambient temperature. The mobile phase was 11% MeOH in 0.02 *M* phosphate buffer, pH 2.8, containing 0.006 *M* 1-octanesulfonic acid sodium salt as an ion-pairing agent. The injection volume was 20 μl, and the flow rate was 1.0 ml/min.

Determination of GSH and GSSG by the Fluorometric Method. The concentration of GSH and GSSG in the liver was measured by the method of Hissin and Hilf with use of *o*-phthaladehyde (OPT) as a fluorescent reagent (17). Mice were killed by cervical dislocation; livers were removed, blotted, weighed, and used immediately. A portion of tissue, usually 500 mg, was homogenized on ice using a Potter-Elvehjem-type Teflon homogenizer. The solution used for homogenization consisted of 7.5 ml of 0.1 *M* phosphate buffer (pH 8.0) containing 0.005 *M* EDTA and 2 ml of 25% HPO_3. The total homogenate was centrifuged at 4°C at 100,000*g* for 30 min to obtain the supernatant.

GSH Assay. To 0.5 ml of the 100,000*g* supernatant, 4.5 ml of the phosphate-EDTA buffer, pH 8.0, was added. The final assay mixture (2.0 ml) contained 100 μl of the diluted tissue supernatant, 1.8 ml of the phosphate-EDTA buffer, and 100 μl of 0.1% OPT methanol solution. After thorough mixing and incubation at room temperature for 15 min, fluorescence at 420 nm was determined with the activation at 350 nm.

GSSG Assay. A 0.5-ml portion of the original 100,000*g* supernatant was incubated at room temperature with 200 μl of 0.04 *M* *N*-ethylmaleimide for 30 min to interact with GSH present in the tissue. To this mixture, 4.3 ml of 0.1 *N* NaOH was added. A 100-μl portion of this mixture was taken for measurement of GSSG, using the procedure outlined above for GSH assay, except that 0.1 *N* NaOH was employed as diluent rather than phosphate-EDTA buffer.

It was confirmed that the determination of GSH and GSSG is not affected by the presence of D-GSH and that neither GSH nor GSSG is liberated from the conjugate during the determination process.

Gel Filtration. Chromatography was performed on a column (1.0 × 47 cm) of Sephadex G-150. A 5-mg sample dissolved in 0.25 ml of 0.2 *M* NaCl solution was applied on the column. Elution was with 0.2 *M* NaCl solution at a flow rate of 10 ml/hr, and 1.0-ml fractions were collected automatically.

In Vitro *Release Experiment.* The release of GSH from the conjugate was determined in 0.1 *M* phosphate buffer (pH 7.4, $\mu = 0.3$) containing 0.002 *M* EDTA at 37°C. The stability of GSH was also carried out under the same conditions. The experiment was initiated by the addition of the stock solution to a preheated buffer solution to give a concentration of 5 mg/ml of D-GSH or 100 μg/ml of GSH, respectively. At fixed time intervals, 0.1 ml of the sample was mixed with the same volume of 1/3 *M* HCl, then 40 μl of the mixture was injected into the HPLC system.

Animal Experiment. Male ddY mice (20–30 g) were obtained from Shizuoka Agricultural Co-operate Association for Laboratory Animals (Shizuoka, Japan). Mice fasted for 24 hr were given D-GSH (0.39 mmol/kg in GSH equivalent) or GSH (0.39 mmol/kg) dissolved in 0.2 ml of saline intravenously through the tail vein without anesthesia. As a control, 0.2 ml of saline was injected instead of the drug solution in all animal experiments. In another study, mice fasted for 24 hr were pretreated with intraperitoneal injection of buthionine sulfoximine (BSO, 2 mmol/kg). After 4 hr, mice were administered the same dose of D-GSH or GSH intravenously. Levels of GSH and GSSG in the liver were determined with the fluorometric method described earlier.

The protective effect of D-GSH or GSH on acetaminophen (APAP)-induced hepatotoxicity was examined as follows. Mice were administered intraperitoneally a lethal dose of APAP (5 mmol/kg) dissolved in 0.2 ml of propylene glycol. Then intravenous injection of D-GSH (0.39 mmol/kg in GSH equivalent) was done both 2 hr before and at the same time as the APAP injection (Method 1), 2 hr before the APAP injection (Method 2), at the same time as the APAP injection (Method 3), or 24 hr after the APAP injection (Method 4). Furthermore, D-GSH (0.39 mmol/kg in GSH equivalent), GSH (0.39 mmol/kg), or dextran (3.4×10^{-5} mol/kg) was administered intravenously to the APAP-treated mice by the way of Method 1. The animals were allowed food and water *ad libitum* and were housed in the standard cage, being observed for 30 days. In this experiment, several mice were sacrificed at 6, 24, and 48 hr after the APAP administration for the determination of serum glutamate pyruvate aminotransferase (SGPT) activity using a kit method (Monotest, Boehringer Mannheim, West Germany). In the case of the determination of GSH and GSSG in the liver, mice were treated with an intravenous injection of a 0.4-ml saline solution of D-GSH (0.98 mmol/kg in GSH equivalent) or GSH (0.98 mmol/kg) both 2 hr before and at the same time as the APAP injection (5 mmol/kg, ip).

RESULTS

D-GSH obtained synthetically was a water-soluble white powder containing 10.3 ± 1.5 (w/w%) of GSH (the average of 77 batches) according to the determination of sulfhydryl group by the method of Ellman. Almost 100% of dextran appeared to be recovered as D-GSH according to the total amount of the conjugate and its GSH content.

Both GSH and D-GSH gave a peak at 218 nm as shown in Fig. 1. Dextran T-40 had no absorption at all, whereas the CNBr-activated dextran showed a significant absorption in the UV region of 200 to 250 nm.

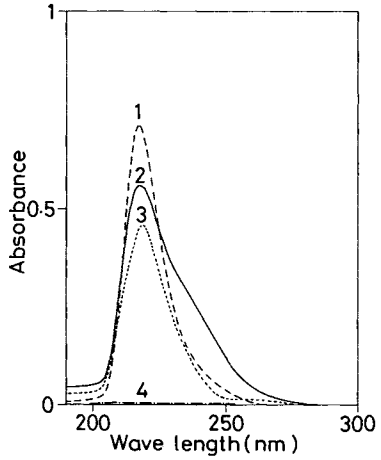


Fig. 1. UV spectra of D-GSH in 0.1 *M* phosphate buffer, pH 7.4. 1, GSH; 2, D-GSH; 3, CNBr-activated dextran; 4, dextran. Measurements on the solutions were made at a concentration of 200 μg/ml.

Figure 2 illustrates typical elution profiles of D-GSH, dextran T-40, GSH, and blue dextran 2000 separated on the Sephadex G-150 column. There was a good agreement between the elution profile of D-GSH followed by the carbohydrate analysis and that measured spectrophotometrically based on the absorption of GSH. When GSH and dextran T-40 were simply mixed and applied to the column, they were eluted separately. These results indicate that GSH is covalently bound to the CNBr-activated dextran. The conjugate eluted somewhat earlier than the original dextran and its elution peak tended to be broadened to some extent. The molecular weight of the peak top was estimated to be 2.5×10^5 by interpolation of a calibration curve of dextran standards.

A representative chromatogram demonstrating the separation of GSH and GSSG is shown in Fig. 3. The retention times were 5.2 and 23.7 min, respectively, and were highly reproducible. Standard curves for GSH and GSSG were linear over the range 0–26 nmol injected. Correlation coefficients of 0.999 were obtained from both. Minimum detectable quantities were 6.5 and 13.0 pmol, respectively. The

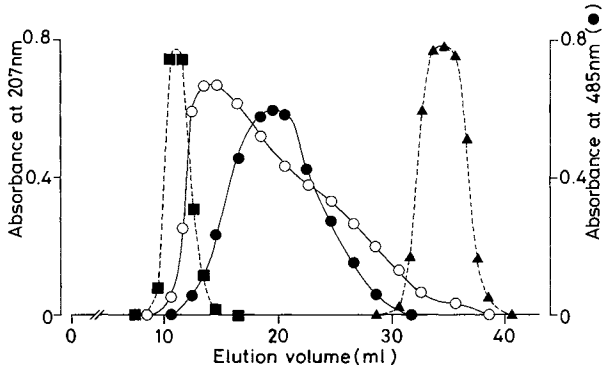


Fig. 2. Elution profiles of D-GSH (○), dextran T-40 (●), GSH (▲), and blue dextran 2000 (■). Gel permeation chromatography was carried out on a Sephadex G-150 column (470 × 10 mm) with 0.2 *M* NaCl at room temperature. D-GSH, GSH, and blue dextran 2000 were spectrophotometrically detected at 207 nm, whereas dextran T-40 was analyzed by the phenol-sulfuric acid method.

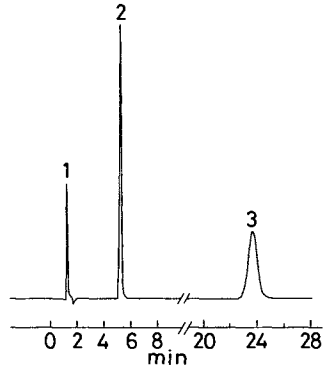
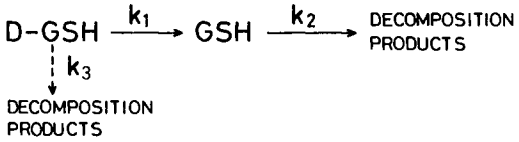


Fig. 3. Reversed-phase high-performance liquid chromatogram of glutathione. Column: 150 × 4.6 mm Cosmosil $5C_{18}$. Mobile phase: 11% MeOH in 0.02 *M* phosphate buffer, pH 2.8, containing 0.006 *M* 1-octanesulfonic acid sodium salt. Flow rate: 1.0 ml/min. Sample size: 20 μl. Detection: UV, 200 nm; range, 0.32. Peaks: 1, solvent effect; 2, GSH (1 μg); 3, GSSG (1 μg).

presence of D-GSH did not disturb the determination of GSH; no GSH was liberated from the conjugate during the analytical process.

The kinetics of release of GSH from the conjugate was examined in aqueous phosphate buffer (pH 7.4) at 37°C. Since the degradation of GSH followed pseudo first-order kinetics, the overall reactions were described by Scheme I, where k_1, k_2, and k_3 are first-order rate constants for the depicted reactions, and Eq. (1).



Scheme I

$$[GSH] = k_1[GSH]^* / [k_2 - (k_1 + k_3)] \times \{\exp[-(k_1 + k_3)t] - \exp(-k_2 t)\} \quad (1)$$

where [GSH]* is the initial concentration of GSH covalently bound to D-GSH. On the other hand, the stability of GSH is expressed by the following equation:

$$[GSH] = [GSH]_0 \exp(-k_2 t) \quad (2)$$

where $[GSH]_0$ is the initial concentration of GSH. These two kinetic equations were simultaneously fitted to observed time courses shown in Fig. 4 using a nonlinear least-squares program (MULTI) (18).

The model suggested in Scheme I, however, suffered from divergence in all computations. However, assuming that the degradation of GSH bound to dextran is negligible ($k_3 \simeq 0$ hr^{-1}), excellent convergence was obtained in the curve fitting using the algorithm of Gauss–Newton method. The first-order rate constants estimated by MULTI were as follows: $k_1 = 0.508 \pm 0.037$ hr^{-1} ($t_{1/2} = 1.36$ hr) and $k_2 = 0.00389 \pm 0.00026$ hr^{-1} ($t_{1/2} = 178$ hr).

Hepatic GSH levels after administration of D-GSH, GSH or saline as a control were examined in mice fasted for 24 hr. The dose of D-GSH was determined by converting to the amount of GSH (0.39 mmol/kg). As shown in Fig. 5, no

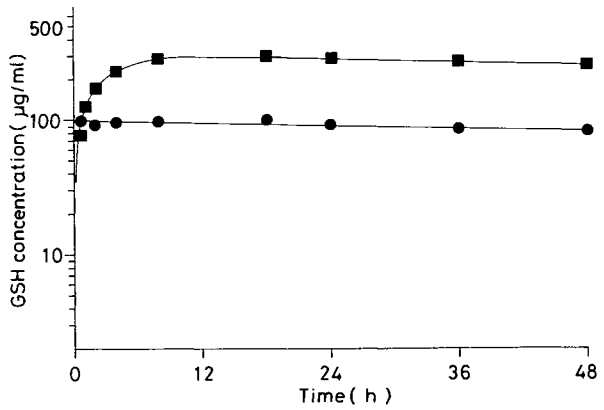


Fig. 4. Stability of GSH (●) and formation of GSH from D-GSH (■) in 0.1 *M* phosphate buffer solution (μ = 0.3) of pH 7.4 at 37°C.

alteration in the hepatic GSH levels was observed in all cases.

Mice fasted for 24 hr were pretreated with BSO, a potent inhibitor of γ-glutamylcysteine synthetase, to prevent intracellular GSH synthesis (19). The GSH level of the liver decreased to one-third of the initial level 4 hr after the administration of BSO (2 mmol/kg) (Fig. 6). Intravenous administration of D-GSH led to a marked increase in the level of GSH. However, administration of free GSH had no significant effect on the hepatic GSH.

Protection against APAP-induced hepatotoxicity by intravenous D-GSH was examined. Mice were treated intraperitoneally with a lethal dose of APAP (5 mmol/kg). Four different treatment regimens of D-GSH were adopted as shown in Fig. 7. The effects of the treatment with D-GSH at a specified time on the survival ratio after the APAP administration are depicted in Fig. 8. When given by the method 1, an intravenous D-GSH (0.39 mmol/kg in GSH equivalent) increased the survival ratio progressively. However, the treatment by method 2 or 3 showed a partial protection; the

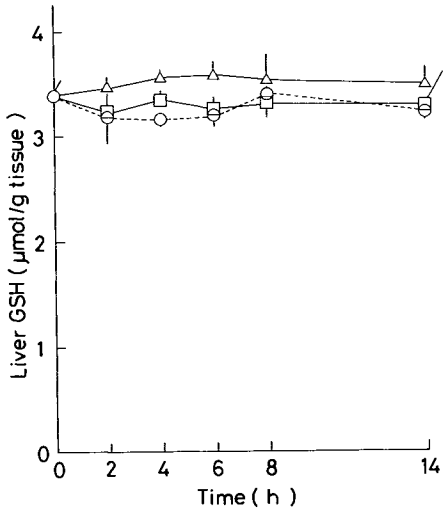


Fig. 5. Effects of D-GSH and GSH on hepatic GSH contents in mice. Mice fasted for 24 hr were given D-GSH (△; 0.39 mmol/kg in GSH equivalent), GSH (□; 0.39 mmol/kg) or saline (○; control) intravenously. Values are given as means ± SE for groups of 5–10 animals.

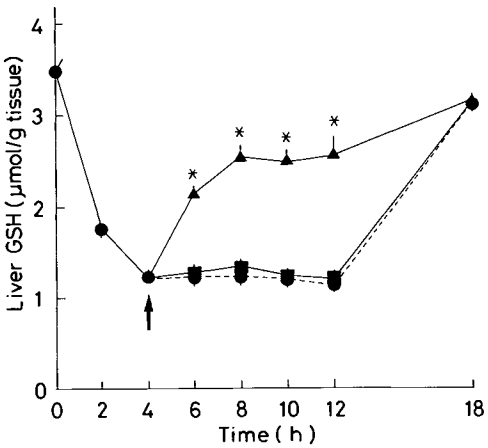


Fig. 6. Effects of D-GSH and GSH on hepatic contents in mice treated with buthionine sulfoximine (BSO). Mice fasted for 24 hr were injected intraperitoneally with BSO (2 mmol/kg). Four hours later (arrow), groups of mice were given D-GSH (▲; 0.39 mmol/kg in GSH equivalent), GSH (■; 0.39 mmol/kg) or saline (●; control) intravenously. Values are given as means ± SE for groups of 5–10 animals. (*) $P < 0.01$ compared with the control. The Cochran-Cox test was used.

treatment by method 4 provided no additional survival effect compared to the treatment of the control.

When given by method 1, the same amount of intravenous GSH (0.39 mmol/kg) showed only little protection against APAP toxicity. The treatment with dextran by the same method provided no survival effect (Fig. 9).

In the experiments described in Fig. 9, blood samples were collected at 6, 24, and 48 hr after the administration of APAP for the determination of SGPT activity. It was markedly increased to the value of 1874 ± 711 U/L at 6 hr after the APAP injection; the SGPT activity of normal mice was 39 ± 4 U/L. As shown in Fig. 10, the treatment with D-GSH maintained the SGPT activity at significantly lower levels (201 ± 50 U/L at 6 hr). However, the treatment with GSH had no suppressive effect on the elevation of the SGPT activity.

Figure 11A shows that the hepatic GSH level at 2 hr after the APAP injection was reduced to 15% of the control level. The intravenous administration of D-GSH led to a significant increase in the level of GSH. Although free GSH also had a significant effect on the recovery of the hepatic GSH level at 2 and 4 hr, it was relatively low compared to that of D-GSH and no longer appreciable at 6 and 8 hr after the APAP administration.

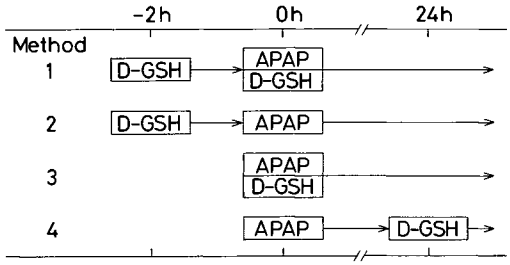


Fig. 7. Treatment regimens of D-GSH on the protection against APAP-induced hepatotoxicity in mice. APAP, 5 mmol/kg, ip; D-GSH, 0.39 mmol/kg in GSH equivalent, iv.

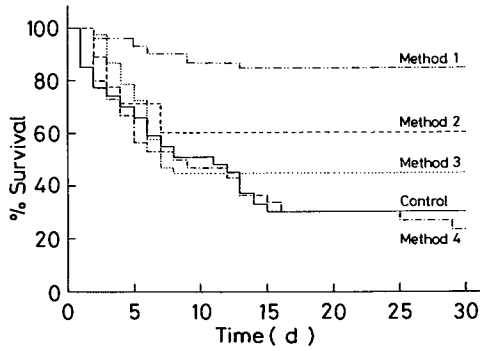


Fig. 8. Survival of mice given a lethal dose of APAP; relationship between time of D-GSH administration and effectiveness. All mice received intraperitoneally a 0.2-ml propylene glycol solution of APAP (5 mmol/kg; control). Mice were then treated by different regimens of intravenous injection of a 0.2-ml saline solution of D-GSH (0.39 mmol/kg in GSH equivalent). See text and Fig. 7 for details.

The hepatic level of GSSG was also reduced by the injection of APAP as shown in Fig. 11B. The treatment with intravenous D-GSH elicited a marked increase in the level of GSSG. However, it was scarcely restored by the administration of free GSH.

DISCUSSION

The change of pH markedly influenced the UV absorption of GSH, which was largely increased and shifted to shorter wavelengths in acidic conditions. This tendency also occurred in D-GSH but not in the CNBr-activated dextran, indicating that the conjugate contains GSH. Furthermore, we made a dextran conjugate of ethanolamine which is usually used to block excess reactive sites of the CNBr-activated dextran. It was found that the conjugate gave a much greater UV absorption than the CNBr-activated dextran. Since ethanolamine has no measurable absorption at pH 7.4 in the ultraviolet region, the linkage of ligands to the CNBr-activated dextran increased UV absorption. Therefore, these findings suggest that the GSH content of D-GSH cannot be accurately estimated by the measurement of UV absorption.

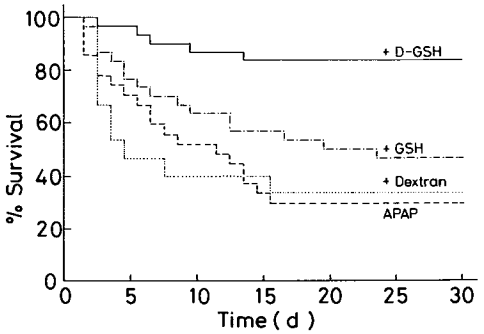


Fig. 9. Survival of mice given a lethal dose of APAP; effect of administration of D-GSH. All mice received intraperitoneally a 0.2-ml propylene glycol solution of APAP (5 mmol/kg). Mice were then treated by intravenous injection of a 0.2-ml saline solution of D-GSH (0.39 mmol/kg in GSH equivalent), GSH (0.39 mmol/kg), or dextran (3.4×10^{-5} mol/kg) according to method 1.

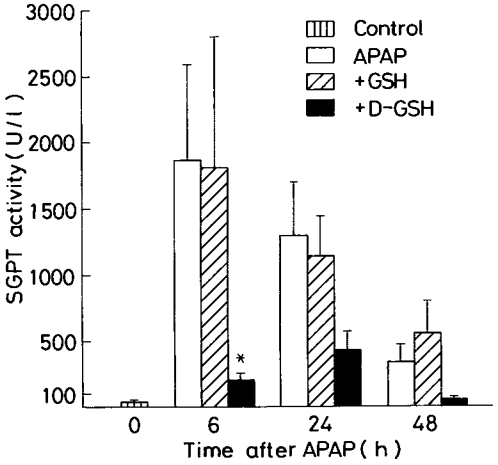


Fig. 10. Time course of SGPT elevation after ip administration of APAP to mice; effect of GSH or D-GSH. At various times after APAP (5 mmol/kg) was given to mice, animals were sacrificed and SGPT activities were determined. Mice were treated by intravenous injection of a 0.2-ml saline solution of GSH (0.39 mmol/kg) or D-GSH (0.39 mmol/kg in GSH equivalent) both 2 hr before and at the same time as APAP administration. Each value represents the mean ± SE of 7–14 mice. (*) $P < 0.05$ compared with the value of APAP. The Cochran-Cox test was used.

Yamamoto and Suzuki (20) have investigated the stability of GSH in aqueous buffer solutions at various pHs. GSH was relatively stable in acidic media, but it was extremely unstable under alkaline conditions. They reported that 55% of GSH remained as such in the buffer solution (pH 5.0) preserved 1 week in the dark at 20°C, and 30% was recovered as GSSG. Matsuki and Sumi (21) have found that GSH decomposed directly into pyroglutamic acid and cysteinylglycine under anaerobic aqueous solution, and above pH 7 desulfurization of GSH took place simultaneously.

As shown in Fig. 4, GSH disappeared slowly under physiological conditions. After incubation for 48 hr, most of the disappeared GSH was detected as GSSG in the medium. However, the decomposition of GSH is quite complicated and depends upon the conditions such as pH, temperature, coexisting substances (O_2, metal ions), concentration and species of buffer, etc. Therefore, we treated the decompositions of GSH as a apparent first-order rate process.

The binding structures of ligands to the CNBr-activated dextran are heterogeneous and quite sophisticated (13,22). No definitive data were obtained from the NMR analysis partly because a sufficiently high concentration of D-GSH in D_2O could not be attained. We carefully examined the contents of free SH groups and free NH_2 groups of D-GSH. These results suggested that GSH mostly binds to the dextran via its sulfhydryl group rather than amino group. This is reasonable because sulfhydryl groups are more reactive than amino groups. Therefore, this may protect GSH against both the autooxidation of the thiol group and the breakdown in the body, then may contribute to the advantageous features of the conjugate as a macromolecular prodrug. Details on these will be reported elsewhere.

Dextran with a molecular weight lower than 15×10^3 is mainly eliminated by the kidney. Larger molecules with a molecular weight above 50×10^3 are practically not excreted

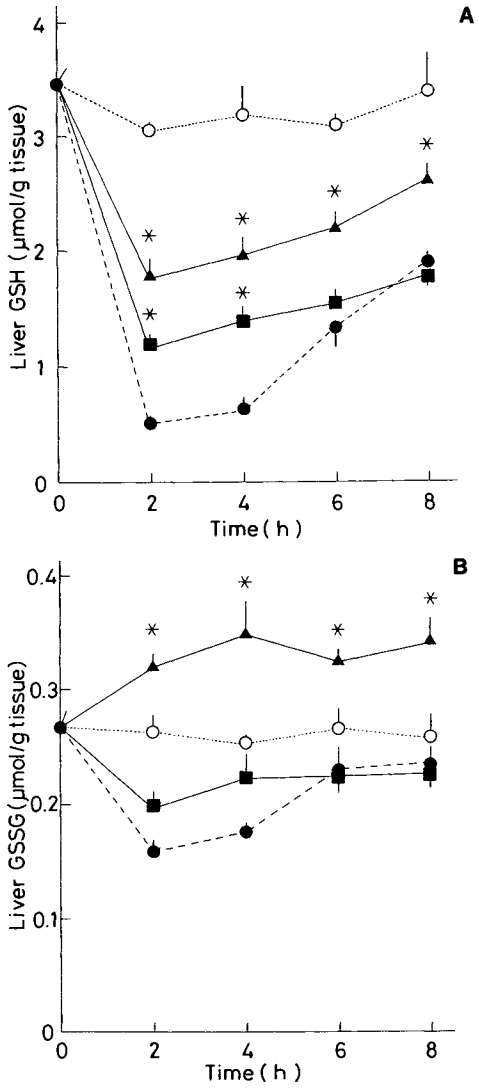


Fig. 11. Effects of D-GSH and GSH on hepatic contents of GSH (A) and GSSG (B) in mice treated with APAP. All mice received intraperitoneally a 0.2-ml propylene glycol solution of APAP (5 mmol/kg). Mice were then treated by intravenous injection of a 0.4-ml saline solution of D-GSH (▲; 0.98 mmol/kg in GSH equivalent), GSH (■; 0.98 mmol/kg), or saline (●) both 2 hr before and at the same time as APAP administration. Control mice (○) received corresponding treatments without the drugs. Each value represents the mean ± SE of 10 mice. (*) $P < 0.01$ compared with the value of the treatment of saline (●). The Cochran-Cox test was used.

via this route (23). However, high molecular weight dextran is completely eliminated from the systemic circulation by biodegradation. In this case dextran uptake by the liver is seen, and a continuous, rapid and complete elimination from the parenchymal cells takes place (24). Both parenchymal and Kupffer cells are shown to participate in the removal of dextran by histological studies in mice (25). However, our present knowledge on the interaction of D-GSH with these cells does not allow further conclusions regarding the site of uptake or distribution.

The results depicted in Figs. 5 and 6 suggest that the conjugate effectively delivers GSH into the hepatic cells only in the GSH-deficient state. Wendel and Jaeschke reported a similar observation that an intravenous injection of liposomally entrapped GSH led to an marked increase in hepatic GSH in the starved mice but had no effect in the fed mice (6). It appears that the liver takes up extracellular GSH only when a certain maximum amount is not yet reached. This may be attributed to the homeostasis which keeps the intracellular GSH level constant (26–29).

APAP is a widely used nonprescription analgesic that produces hepatotoxicity and death in animals and humans when large doses of this drug are administered. High doses of APAP deplete hepatic GSH and suppress the biosynthesis of GSH (30,31). According to the established concept, the hepatotoxic entity of APAP is its reactive metabolite, *N*-acetyl-*p*-benzoquinone imine, which covalently binds to macromolecules (32–34) and conjugated by GSH (34). However, activated oxygen such as O_2^- or H_2O_2 released during redox metabolism of APAP is responsible for the observed lipid peroxidation accompanied by severe acute liver necrosis (6,35–40). Glutathione peroxidase catalyzes the reduction of lipid hydroperoxides and hydrogen peroxide to alcohols and water (1). Wendel and Feuerstein have demonstrated that this selenium-dependent enzyme protects the liver against the oxidative damage evoked by the phase I metabolism of APAP, as long as sufficient GSH is available (37).

Earlier reports indicated that significant tissue necrosis is not seen until hepatic GSH has been depleted to 20–30% of normal (41–43). As shown in Fig. 11A, the hepatic level of GSH fell to 15% of the normal state 2 h after the administration of sufficiently high (hepatotoxic) dose of APAP. Intravenous injection of D-GSH led to a 3.3-fold increase in the level of GSH which reached 50% of the initial level. The treatment with free GSH showed a less pronounced effect.

The hepatic content of GSSG was decreased by the administration of APAP as well as that of GSH (Fig. 11B). It was increased to 120–130% of the initial control level by treatment with D-GSH. The control mice gave GSH:GSSG ratios ranging from 10.0 to 14.5; the average value of 20 mice was 12.3 ± 1.6. This ratio dropped significantly, to 3.2–8.1, upon administration of APAP. A significant decrease in the ratio was also observed in the animals treated with GSH or D-GSH; the ratios were 6.0–7.9 and 5.6–7.7, respectively. Since hepatocytes normally maintained a physiologic balance, the change in the GSH:GSSG ratio suggests that GSH is more rapidly converted to GSSG because of the reduction of reactive oxygen species by glutathione peroxidase (39). The present study does not allow us to determine quantitatively whether extracellularly delivered GSH acts as a nucleophile as in the case of its reaction with *N*-acetyl-*p*-benzoquinone imine or as a cofactor for glutathione peroxidase. Our results, however, suggest that D-GSH protects hepatic cells against the toxic effects of APAP by keeping the hepatic level of GSH above the critical value, especially in the initial stage of the toxic insult.

N-Acetylcysteine and other sulfhydryl donors such as cysteine and methionine have been shown to be effective antidotes protecting against the hepatotoxicity of APAP both in animal models and clinical practice. Because of its low toxicity, *N*-acetylcysteine is currently the favorite compound in APAP intoxications (44). Earlier reports indicated that these antidotes share a common mechanism of action in protecting against the APAP toxicity, namely, facilitation of

GSH synthesis (45,46). This suggests that GSH would be an excellent antidote against APAP poisoning if it penetrates into hepatocytes.

It was found that D-GSH effectively protected mice against APAP acute poisoning resulting from hepatotoxicity whereas free GSH had no effect (Figs. 9 and 10). BSO, a potent inhibitor of γ-glutamylcysteine synthetase, prevents the intracellular de novo synthesis of GSH from its constituent amino acids. The studies in which mice were pretreated with BSO (Fig. 6) strongly suggested that D-GSH is taken up into hepatic cells and liberates GSH.

The present findings may provide a new approach to increase the intracellular GSH levels of tissues and a clinically effective means of augmenting hepatic free thiol content in the event of GSH depletion due to toxic insult.

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