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Preparation and characterization of a soluble glutathione-dextran conjugate

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Summary

Glutathione (GSH), a naturally occurring antidote, cannot permeate into the liver when given extracellularly and has a very short half-life in the body. A dextran conjugate of GSH was synthesized by coupling GSH covalently to dextran (T-40, $M_w = 43{,}900$) by the CNBr activation method in order to improve these disadvantages. We have demonstrated that GSH is delivered effectively into the hepatic cells by the conjugate which protects mice from the acetaminophen hepatotoxicity (Kaneo, Y. et al., *Int. J. Pharm.*, 44 (1988) 265–267). The conjugate was a water-soluble white powder containing 10% w/w of GSH. The molecular weight of the conjugate was distributed more widely than the original dextran and that of the peak estimated by size exclusion chromatography was 2.5×10^5. The isoelectric point of the conjugate was estimated to be 2.5 by the cellulose acetate paper electrophoresis. Kinetics of GSH regeneration from the conjugate was examined at various pH values. The conjugate significantly stabilized GSH and liberated it gradually at physiological conditions ($t_{1/2} = 99$ min). Tripeptide GSH has one sulfhydryl, one amino, and two carboxyl groups in the molecule. It was found that the sulfhydryl groups participate in chemical bonding between GSH and CNBr-activated dextran and appear gradually by the cleavage of the bonding according to the determination of sulfhydryl groups by the method of Ellman. It was confirmed that the content of free amino groups of the conjugate is very high by the measurement of amino groups with 2,4,6-trinitrobenzenesulfonic acid (TNBS). These results indicated that at least 80% of the conjugated GSH are attached to dextran via sulfhydryl groups. This may contribute to chemical stability against autoxidation of the thiol group and then to the advantageous features of the conjugate as a macromolecular prodrug.

Introduction

Tripeptide glutathione (GSH) is one of the major detoxication factors of the liver. It plays an important role in the protection against both free radicals and reactive oxygen compounds (Meister and Anderson, 1983). Since GSH is a naturally occurring sulfhydryl compound, it would be an excellent antidote against these hepatotoxins. However, extracellular GSH cannot permeate into the liver and has a very short half-life due to its rapid renal degradation into the constituent amino acids (Hahn et al., 1978; Griffith and Meister, 1979; Wendel and Jaeschke, 1982).

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In an attempt to improve these disadvantages, we have synthesized a dextran-GSH conjugate (D-GSH) by coupling GSH covalently to dextran, a clinically acceptable polysaccharide that has a high loading capacity due to the presence of the large amount of hydroxy groups available for derivatization. In a preliminary study (Kaneo et al., 1988), it was demonstrated that GSH is delivered effectively into the hepatic cells by the conjugation to dextran and it protects mice from acetaminophen hepatotoxicity. The present report presents details of the preparation and properties of D-GSH and describes proposed chemical bonds between GSH and dextran molecules.

Materials and Methods

GSH was obtained from Sigma, St. Louis. Dextran T-40 ($M_w = 43{,}900$ and $M_n = 26{,}200$) was purchased from Pharmacia, Sweden. All other chemicals were of analytical grade or the best quality.

Preparation of dextran-GSH conjugate

GSH was conjugated to dextran using the cyanogen bromide (CNBr) activation method (Axén and Ernback, 1971). In short, 0.2 g of dextran was dissolved in 20 ml of water at ambient temperature and 110 mg of CNBr in 1.5 ml of water was added dropwise under vigorous stirring. The pH was maintained at 11.0 by addition of 4 M NaOH until the pH had stabilized. The pH was then adjusted to 6.5 by addition of 0.1 M HCl and 0.4 g of GSH was added while the pH was maintained at 6.5. The coupling of GSH to CNBr-activated dextran was allowed to proceed for 18 h at 4 °C. The reaction mixture was washed 9 times with 0.1 M CH_3COOH by pressure dialysis using a UHP-43 ultrafiltration cell incoporating a UP-20 membrane (Toyo, Tokyo, Japan) and was finally concentrated to a volume of 10 ml. Excess reagents and low molecular by-products of the reaction were removed by this process. The dextran-GSH conjugate was obtained from lyophilization of the final solution. Dextran was recovered in an almost 100% yield.

Synthesis of N-acetylglutathione

N-Acetylglutathione (AcGSH) was prepared by the method of Anderson et al. (1985). GSH (1 g) was dissolved in 3 ml of formic acid and 1.5 ml of acetic anhydride was added at room temperature. The reaction, followed by thin-layer chromatography, was completed in 2 h. A large excess of diethyl ether was added to precipitate the product, which was separated, washed with ether, and dried in vacuo. The crude product was recrystallized from dimethyl formamide : ethyl acetate at 0 °C twice; yield, 0.7 g; m.p., 82–91 °C. Calc. for $C_{12}H_{19}N_3O_7S \cdot C_3H_7NO$: C, 42.65; H, 6.22; N, 13.26. Found: C, 42.18; H, 6.16; N, 13.50. The product gave 92.3% of the theoretical reaction with 5,5′-dithiobis(2-nitrobenzoate) (DTNB).

Determination of degree of substitution

The degree of substitution was estimated by measurement of the sulfhydryl group according to the method of Ellman (1959). In short, 3.96 mg of DTNB was dissolved in 1 ml of a phosphate buffer (pH 7.0, $\mu = 0.1$). The DTNB reagent (0.02 ml) was added to 3.0 ml of the sample 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 $M^{-1} \cdot cm^{-1}$.

Determination of amino groups

The free amino groups of GSH conjugated to dextran were quantitated using 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Habeeb, 1966; Fields, 1971, 1972). The sample containing amino groups was added to 0.2 ml of a borate buffer (0.1 M $Na_2B_4O_7$ in 0.1 M NaOH) and the volume was made up to 0.4 ml. Then, 0.008 ml of a solution of 1.1 M TNBS was added and the solution was rapidly mixed. After 5.0 min the reaction was stopped by adding 0.8 ml of 0.1 M NaH_2PO_4 which contained 1.5 mM Na_2SO_3, and the absorbance at 420 nm was determined. Values for the molar absorptivity of α-amino groups of GSH were 18,510 $M^{-1} \cdot cm^{-1}$, and for thiol groups, 2,061 $M^{-1} \cdot cm^{-1}$. These values were obtained by measuring the color development of GSH (α-NH_2 + SH) and AcGSH (SH).

Determination of carbohydrate

Dextran was measured by the phenol-sulfuric acid method (Dobois et al., 1956), calibrated against glucose. As it was pointed out (Marshall and Rabinowitz, 1976), CNBr 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.

Characterization of the conjugate

Two kinds of chromatographies were carried out to characterize the conjugate. Size exclusion chromatography was performed on a column (1.0 × 47 cm) of Sephadex G-150. A 5 mg of sample dissolved in 0.25 ml of 0.2 M NaCl was applied on the column. Elution was with 0.2 M NaCl at a flow rate of 10 ml/h, and fractions (1 ml) were collected automatically. The elution volume of the conjugate was determined by analysing fractions for UV absorption at 207 nm and the elution of dextran was followed using the carbohydrate analysis.

Size exclusion chromatography was carried out using a Tosoh (Tokyo, Japan) HPLC system (CCPD) equipped with a variable-wavelength UV detector (SPD-6A, Shimadzu, Kyoto, Japan) operated at 204 nm and a differential refractometer (RI-8000, Tosoh). A 7.5 × 600 mm, TSK gel G3000SW column (Tosoh) was used at ambient temperature. The mobile phase was 0.2 M NaCl in 0.05 M phosphate buffer, pH 7.0. The injection volume was 80 μl, and the flow rate was 1.0 ml/min.

Determination of the isoelectric point

Cellulose acetate paper electrophoresis was performed to determine the isoelectric point of the conjugate following the procedure of Kubota and Ueki (1968). Bovine serum albumin (BSA) was used as a standard substance ($pI = 4.7$). D-Glucose was employed for correcting the migration caused by electroosmosis. The buffers used were HCl-glycine solutions, pH 2.0–3.5 and acetate solutions, pH 4.0–5.5. A constant ionic strength of 0.1 was maintained for each buffer by adding a calculated amount of sodium chloride. D-GSH and BSA were stained with 0.8% Ponceau 3R containing 6% trichloroacetic acid. The band of D-glucose was detected with 1% of potassium permanganate containing 2% sodium carbonate.

Determination of GSH by the fluorometric method

The determination of GSH and oxidized GSH (GSSG) was performed by a modification of the method of Hissin and Hilf (1976) with use of *o*-phthaldehyde (OPT) as a fluorescent reagent.

GSH assay. Samples were prepared each time in 0.005 M EDTA solution and used immediately. To a mixture of 1.8 ml of 0.1 M phosphate buffer (pH 8.0) containing 0.005 M EDTA and 0.1 ml of a methanol solution of 0.1% OPT, 0.1 ml of the sample solution was added. After thorough mixing and incubation at room temperature for 30 min, fluorescence at 420 nm was determined with the activation at 350 nm.

GSSG assay. A 0.5-ml portion of the sample solution was incubated at room temperature with 0.2 ml of 0.04 M *N*-ethylmaleimide for 30 min to interact with GSH present. To this mixture, 4.3 ml of 0.1 N NaOH was added. A 0.1-ml 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.

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 (Kyoto, Japan) liquid chromatographic system (LC-6A) with a variable-wavelength UV detector (SPD-6A) operated at 200 nm. A 4.6 × 150 mm, 5-μm particle size, C_{18} reversed-phase column (Cosmosil $5C_{18}$, Nakarai, Kyoto, Japan) was used at ambient temperature. The mobile phase was 7% 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 40 μl, and the flow rate was 1.0 ml/min. Under these conditions, GSH was eluted with a retention time of 6 min. It was confirmed that the determination of GSH is not affected by the presence of D-GSH and no GSH is liberated from the conjugate during the determination process.

In vitro release experiment

The release of GSH from the conjugate was determined in a phosphate buffer system (pH 5.0–9.0, 0.1 M, $\mu = 0.3$) 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 a fixed time interval, 0.1 ml of the sample was withdrawn and mixed with the same volume of 1/3 M HCl, then 40 μl of the mixture was injected into the HPLC system.

Microultrafiltration

Free GSH or low molecular materials were separated from the sample solution using a Minicent-10 ultrafiltration system (Tosoh, Tokyo, Japan) which cuts off the materials below 10,000 of molecular weight.

Reduction of D-GSH by $NaBH_4$

In order to estimate the content of GSSG in the conjugate, the disulfide bond was reduced. To 0.5 ml of a sample solution, 15 mg of $NaBH_4$ was added. After mixing and incubation at room temperature for 60 min, the mixture was acidified to decompose excess $NaBH_4$ by addition of 2 ml of 0.1 N HCl. Then, 0.5 ml of the mixture was diluted to 10 ml with 0.1 M phosphate buffer (pH 8.0). The sulfhydryl group was determined by the method of Ellman described earlier. In the control experiment, 77.5 ± 2.4% of GSSG was recovered as reduced glutathione (GSH).

Results and Discussion

Preparation of dextran-GSH conjugate

The conjugate was a water-soluble white powder containing 10.0 ± 0.6% w/w of GSH (the average of 112 batches) according to the determination of sulfhydryl group by the method of Ellman. The degree of substitution by GSH of the dextran was estimated to be one molecule per approximately 17 glucose units. In all cases the reaction product was completely soluble in water.

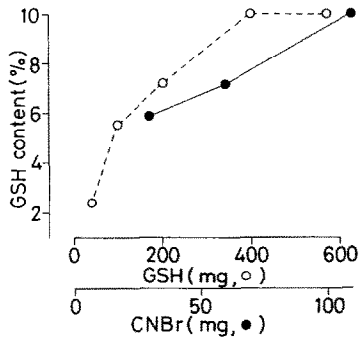


Fig. 1. Effect of the amounts of reagents on the GSH content in D-GSH. The amounts of CNBr and GSH added were fixed at 110 and 400 mg, respectively, in each series of experiments where 0.2 g of dextran was used.

A variety of methods for chemical fixation of drugs to dextrans have been reported (Molteni, 1979; Hurwitz et al., 1980; Larsen and Johansen, 1985). One of the simplest of these methods was selected in the present study. After activation of dextran by treatment with CNBr, addition of GSH resulted in a covalent linkage between the activated monosaccharide residues and the functional groups of GSH. The methods adopted for the activation of insoluble polysaccharides by CNBr (Axén and Ernback, 1971; Schnaar et al., 1977) caused irreversible precipitation of the dextran possibly due to cross-linkage. An essential feature of the activation of soluble dextran is the relative low concentration of CNBr used. As shown in Fig. 1, the content of GSH linked to dextran (% w/w) was increased linearly with the amount of CNBr used. However, immediate precipitation of dextran took place during the activation procedure by addition of more than 110 mg of CNBr. The content of GSH also depended upon the amount of GSH, but did not exceed the value of 10% even if more GSH was added (Fig. 1). Therefore, the conditions that gave the highest degree of GSH linkage without precipitation of the dextran were adopted as described in Materials and Methods.

Characterization of the conjugate

The UV absorbance of GSH depended largely upon pH and the absorption band was shifted to shorter wavelengths in acidic conditions. This tendency could also be seen in D-GSH, indicating

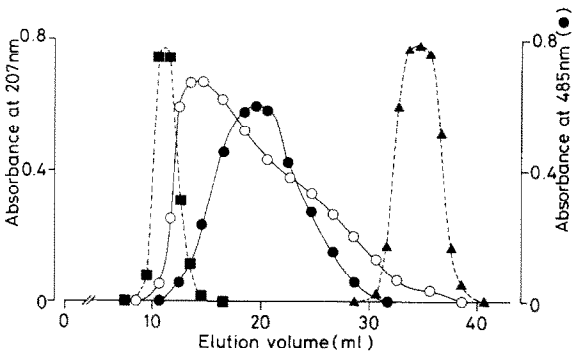
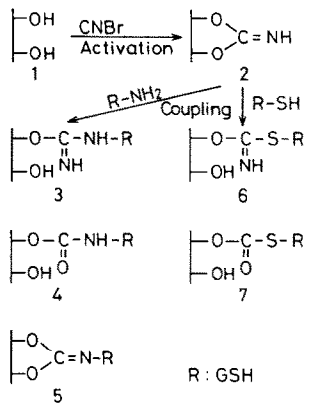


Fig. 2. Elution profiles of D-GSH (○), dextran T-40 (●), GSH (▲), and blue dextran 2000 (■). Size exclusion chromatography was carried out on a Sephadex G-150 column (10×470 mm) with 0.2 M NaCl at room temperature. D-GSH, GSH and blue dextran 2000 were spectrophotometrically detected at 207 nm, whereas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

that the conjugate contains GSH. Dextran T-40 had no absorption at all. However, it was found that the CNBr-activated dextran has absorption in the UV region of 190–250 nm. Therefore, it was pointed out that the accurate GSH content cannot be estimated from the measurement of UV absorption.

Fig. 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 peak of D-GSH followed by the carbohydrate analysis and that measured spectrophotometrically based on the absorption of GSH. The size exclusion chromatography of the conjugate was also performed by HPLC on a TSK gel G3000SW column. Elution peaks were detected simultaneously by both a differential refractometer and a spectrophotometer. When GSH and dextran T-40 were simply mixed and applied to these chromatographic systems, they were eluted at each position separately. These results indicate that GSH is bound covalently to the CNBr-activated dextran in the D-GSH. It was observed that the conjugate eluted somewhat earlier than the original dextran and its elution peak tended to be broadened to some extent. The molecular weight corresponding to the peak estimated by interpolation of a calibration curve of dextran standards chromatographed on the Sephadex G-150 column was 2.5×10^5.



Scheme 1.

The isoelectric point of the dextran-GSH conjugate was determined by cellulose acetate paper electrophoresis. The treatment of polysaccharides by means of CNBr followed by the conjugation of the ligands ($R\text{-}NH_2$) results in the formation of three different covalent linkages such as *N*-substituted isoureas (**3**), *N*-substituted carbamates (**4**) and *N*-substituted imidocarbonate (**5**) as shown in Scheme 1 (Axén and Ernback, 1971). The CNBr activation introduces cationic charge into the conjugate primarily due to formation of **3** and **5** which are expected to carry a positively charged nitrogen at physiological pH (Jost et al., 1974). Fig. 3 shows net migration distance-pH curves obtained from D-GSH and BSA (standard substance). The isoelectric point was estimated as an intersecting point of the curve and the abscissa. As can be seen in Fig. 3, the value was 2.5 which

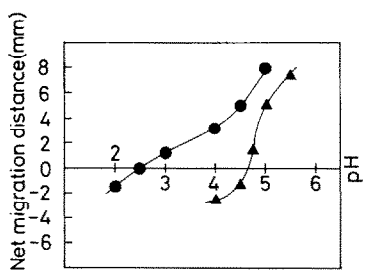


Fig. 3. Effect of pH on the net migration distance of D-GSH (●) and bovine serum albumin (▲). Electrophoresis was run on the cellulose acetate paper at 0.6 mA/cm for 40 min.

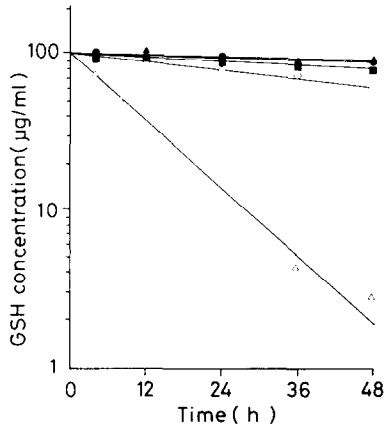


Fig. 4. Stability of GSH in 0.1 M phosphate buffer solutions ($\mu = 0.3$) of pH 5.0 (●), 6.0 (▲), 7.4 (■), 8.0 (○), and 9.0 (△) at 37 °C. The lines are calculated from Eq. 2.

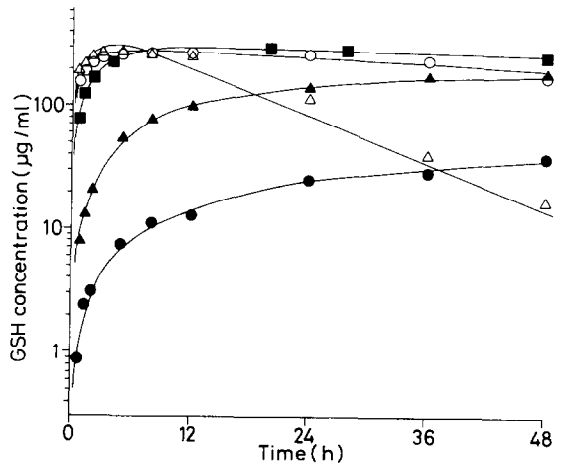
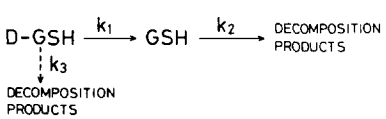


Fig. 5. Formation of GSH from D-GSH in 0.1 M phosphate buffer solutions ($\mu = 0.3$) of pH 5.0 (●), 6.0 (▲), 7.4 (■), 8.0 (○), and 9.0 (△) at 37 °C. The curves are calculated from Eq. 3.

was very low in contrast to the prediction. This is thought to be due to the attachment of GSH which shows relatively high acidity (pH 1–2) in the aqueous solution, having two carboxyl groups ($pK_{COOH(1)} = 2.12$, $pK_{COOH(2)} = 3.53$).

Kinetics of regeneration of GSH from the conjugate

The degradation of GSH was investigated in aqueous phosphate buffer solutions over the pH range 5.0–9.0 at 37 °C. At each pH value the degradation of GSH followed pseudo first-order kinetics as shown in Fig. 4. Time courses for GSH regenerated after incubation of D-GSH in the buffer solutions are shown in Fig. 5. As can clearly be seen at higher pH values, the liberated GSH decomposed simultaneously in this system. On the basis of these findings, the overall reactions, i.e. regeneration and decomposition, may be described by Scheme 2, where k_1, k_2 and k_3 are apparent first-order rate constants for the depicted reactions.



Scheme 2.

Then the concentration of GSH ([GSH]) has a time dependence given by the following equation:

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

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. At first the parameters of k_2 were calculated from the observed time courses shown in Fig. 4 using Eq. 2. Eq. 1 was then fitted to the observed time courses shown in Fig. 5 using a nonlinear least squares program (MULTI) (Yamaoka et al., 1981). In this computation, the values of k_2 were fixed to those obtained separately from Fig. 4.

The model suggested in Scheme 2 suffered the divergence in most of the computations; even if it was converged, the negative value of k_3 with a extraordinarily large standard deviation was obtained. When it was assumed that the degradation

TABLE 1

Estimated conversion rate constants of D-GSH and GSH in 0.1 M phosphate buffer solution (37 °C, $\mu = 0.3$)

pH	Formation rate constant of GSH from D-GSH (k_1, h^{-1})	Decomposition rate constant of GSH (k_2, h^{-1})
5.0	0.0214 ± 0.0057	0.00232 ± 0.00033
6.0	0.0540 ± 0.0038	0.00187 ± 0.00029
7.4	0.421 ± 0.042	0.00389 ± 0.00026
8.0	0.786 ± 0.120	0.0103 ± 0.0006
9.0	0.527 ± 0.110	0.0818 ± 0.0056

Each value is the mean ± S.D. calculated from 15–20 sets of experimental data by the least square method.

of GSH bound to dextran is negligible, however, the excellent convergence could be obtained in the curve fitting using the algorithm of Gauss-Newton method where the following equation was adopted instead of Eq. 1.

$$[GSH] = k_1[GSH]^*/(k_2 - k_1) \times (\exp(-k_1 t) - \exp(-k_2 t)). \quad (3)$$

The apparent first-order rate constants, k_1 and k_2, estimated by the curve fitting are summarized in Table 1. The pH-rate profiles of k_1 and k_2 showed almost parallel curves in the investigated pH range. GSH was relatively stable at acidic pH, but the decomposition increased markedly above neutral pH. The regeneration rate of GSH was also enhanced with a rise in pH and reached a maximum at pH 8. At pH 7.4, the regeneration of GSH was 100-times faster than the degradation of GSH, showing half-lives of 1.6 and 178 h, respectively (Table 1). These findings indicate that covalent binding to dextran stabilizes GSH ($k_3 \simeq 0$) and the macromolecular prodrug, D-GSH, liberates GSH gradually at physiological conditions.

Proposed structures of the linkage of GSH to the CNBr-activated dextran

DTNB reagent react with aliphatic thiol compounds at pH 8.0 to produce one mole of 3-carboxy-4-nitrothiophenol which is highly colored. The molar extinction coefficient at 412 nm (ϵ_{412}) of GSH was 13990 $M^{-1} \cdot cm^{-1}$ which was in fair agreement with the value ($\epsilon_{412} = 13600$ $M^{-1} \cdot cm^{-1}$) reported by Ellman (1959). GSH reacted immediately, indicating such thiols are fully reactive, and kept a steady absorbance for at least 24 h. However, the reaction of D-GSH with DTNB followed a different profile; the color initially developed instantaneously (portion A), then increased gradually until reaching a steady state (portion C). There are at least two possible interpretations of this phenomenon. One is that the macromolecules interfere with the reaction of thiol groups with DTNB and the other possibility is that the sulfhydryl groups participate in chemical bonding between GSH and CNBr-activated dextran and appear gradually by chemical cleavage.

It was confirmed that coexistent dextran (~ 10 mg/ml) does not affect the color development. Furthermore, it was found that AcGSH, whose amino residue is blocked by *N*-acetylation, also linked covalently to the CNBr-activated dextran and the conjugated product (D-AcGSH) showed the same color developing pattern on reaction with DTNB as D-GSH. These findings suggest that the majority of the sulfhydryl groups are responsible for the bonding between GSH and dextran.

GSH contents or AcGSH contents of the conjugates estimated by the measurement of the sulfhydryl groups with the method of Ellman (1959) are summarized in Table 2. The total content (C) of GSH in D-GSH and that of AcGSH

TABLE 2

GSH content of dextran conjugates estimated by the DTNB method [a]

Fraction [b]	D-GSH (w/w%)	D-AcGSH (w/w%)
A	1.46 ± 0.19	1.09 ± 0.19
B	8.58 ± 0.45	7.92 ± 0.93
C	10.0 ± 0.6	9.01 ± 0.94
Ultrafiltrate [c]	0.42 ± 0.08	0.97 ± 0.22

[a] The amount of GSH was estimated from the sulfhydryl content.

[b] A + B = C, see text for details.

[c] Free GSH was separated by the ultrafiltration. Results are means of 112 batches for D-GSH or of 10 batches for D-AcGSH with standard deviations.

TABLE 3

Determination of amino groups and sulfhydryl groups of D-GSH with TNBS [a]

Conc. of D-GSH (μg/ml)	Absorbance at 420 nm			Conc. of free GSH (μM)	Conc. of conjugated GSH [d] (μM)	Conc. of [e] amino group (μM)	Conc. of [e] sulfhydryl group (μM)
	Total	Ultra-filtrate [b]	Conjugate [c]				
32.0	0.158 ±0.009	0.014 ±0.001	0.144 ±0.008	0.682 ±0.049	9.75 ±0.47	7.55 ±0.44	2.20 ±0.19
86.0	0.434 ±0.011	0.039 ±0.001	0.395 ±0.012	1.88 ±0.05	26.1 ±0.2	20.7 ±0.7	5.59 ±0.66

[a] 2,4,6-Trinitrobenzenesulfonic acid.
[b] Free GSH was separated by the ultrafiltration.
[c] Conjugate = Total − Ultrafiltrate.
[d] Conc. of conjugated GSH was estimated by subtracting the free GSH fraction from the level of the total GSH which was measured by the DTNB method.
[e] Concentrations of both groups were calculated by the simultaneous equations (see text for details).
Results are means of five separate experiments with standard deviations.

in D-AcGSH were 10.0 and 9.01% w/w, respectively. The portion A of D-GSH (14.6% of the total content) was found by the ultrafiltration method to contain a small quantity of free GSH (4.2%) which was confirmed by both the HPLC and the OPT methods. On the other hand, portion A of D-AcGSH (12.1% of the total content) consisted solely of free AcGSH. These results indicate that 10% of GSH are linked by a C-N bond to dextran in D-GSH, whereas such a bonding is not available in D-AcGSH because AcGSH has no free amino residue. It is well known that the substitution of the polysaccharide is achieved by CNBr activation, and the ligand is subsequently coupled to the activated site via a free amino group. The coupling is thought to result in *N*-substituted isoureas (**3**), *N*-substituted carbamates (**4**) and *N*-substituted imidocarbonates (**5**) as shown in Scheme 1 (Axén and Ernback, 1971). In the case of GSH, free sulfhydryl groups are more important in the formation of the conjugate and the derivatives of **6** and **7** could be presented as possible structures (Scheme 1).

The amino group determination of D-GSH was performed by the TNBS method. TNBS reacted with both amino and thiol groups of GSH and their molar absorptivities at 420 nm were 18 510 and 2061 $M^{-1} \cdot cm^{-1}$, respectively. Then, if we know the total absorbance of D-GSH reacted with TNBS ($A_{420\ nm}$) and the concentration of GSH bound to dextran (C_{GSH}), the molar concentrations of either amino groups (X) and thiol groups (Y) could be calculated theoretically by the following simultaneous equations.

$$18\,510\ X + 2061\ Y = A_{420\ nm}, \tag{4}$$

$$X + Y = C_{GSH}. \tag{5}$$

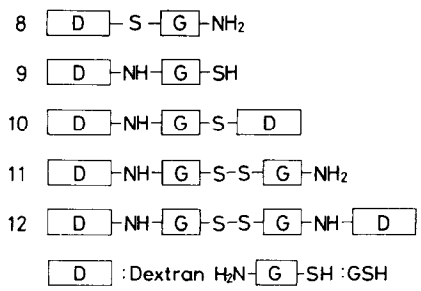
The data are summarized in Table 3. The concentration of conjugated GSH (C_{GSH}) was estimated by subtracting the free GSH fraction from the level of the total GSH which was obtained by the DTNB method. As can be seen in Table 3, the

TABLE 4

Proportion of the types of conjugation estimated by the methods of DTNB and TNBS

Type	Proportion (%)	
	DTNB	TNBS
GSH bound through:		
Sulfhydryl group	89 ± 7	78 ± 2
Amino group	11 ± 5	22 ± 2

Results are means of 112 measurements by the DTNB method or of 10 measurements by the TNBS method with standard deviations.



Scheme 3.

concentration of amino groups was considerably higher than that of sulfhydryl groups. These results are in good agreement with those obtained by the DTNB method, suggesting that GSH mainly binds via the sulfhydryl groups.

The proportions of the different types of linkage between GSH and dextran, estimated by the DTNB and TNBS methods, are summarized in Table 4. Although the TNBS method estimated the linkage through sulfhydryl groups to be a little smaller than that by the DTNB method, at least 80% of the conjugated GSH was found to be attached to dextran via the sulfhydryl groups.

Three more types (**10–12**) of linkage could be suggested besides those described above, see in Scheme 3. The proportion of structure **10** has not been estimated separately so far, however, it would contribute to the overestimation of linkage via sulfhydryl groups in the DTNB method.

It is also conceivable that D-GSH contains GSSG in the forms (**11** or **12**) shown in Scheme 3, as an increase of sulfhydryl groups was observed after the reduction of D-GSH by $NaBH_4$. During these experiments, D-GSH was found to include 4.4% of GSSG as the conjugate type.

The chemical structures of GSH bonding to the CNBr-activated dextran are heterogeneous and quite complex. Very little instructive information was obtained by the NMR analysis partly because a sufficiently high concentration of D-GSH in D_2O could not be attained. However, it is evident that GSH is attached to dextran via sulfhydryl groups. Although the stabilities of C-N and C-S bonds between GSH and dextran have not been examined separately so far, the latter would be easily cleaved under physiological conditions considering that sulfhydryl groups are more reactive than amino groups. It may be concluded that these advantageous features of D-GSH as a macromolecular prodrug are endowed by the highly reactive sulfhydryl groups which play a very important role in a variety of GSH functions.

An investigation focusing on the chemical structure of the linkage and its effects on the nature of the conjugate as a prodrug, is in progress.

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References

Anderson, M.E., Powrie, F., Puri, R.N. and Meister, A., Glutathione monoethyl ester: preparation, uptake by tissues, and conversion to glutathione. *Arch. Biochem. Biophys.*, 239 (1985) 538–548.

Axén, R. and Ernback, S., Chemical fixation of enzymes to cyanogen halide activated polysaccharide carriers. *Eur. J. Biochem.*, 18 (1971) 351–360.

Dubois, M., Gills, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F., Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, 28 (1956) 350–356.

Ellman, G.L., Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, 82 (1959) 70–77.

Fields, R., The measurement of amino groups in proteins and peptides. *Biochem. J.*, 124 (1971) 581–590.

Fields, R., The rapid determination of amino group. *Methods Enzymol.*, 25 (1972) 464–468.

Griffith, O.W. and Meister, A., Glutathione: Interorgan translocation, turnover, and metabolism. *Proc. Natl. Acad. Scl. USA* 76 (1979) 5606–5610.

Habeeb, A.F., Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem.*, 14 (1966) 328–336.

Hahn, R., Wendel, A. and Folhé, L., The fate of extracellular glutathione in the rat. *Biochim. Biophys. Acta*, 539 (1978) 324–337.

Hissin, J.P. and Hilf, R., A fluorometric method for determination of oxidized and reduced glutathione in tissue. *Anal. Biochem.,* 74 (1976) 214–226.

Hurwitz, E., Wilchek, M. and Pita, J., Soluble macromolecules as carriers for daunorubicin. *J. Appl. Biochem.*, 2 (1980) 25–35.

Jost, R., Miron, T. and Wilchek, N., The mode of adsorption of proteins to aliphatic and aromatic amines coupled to cyanogen bromide-activated agarose. *Biochim. Biophys. Acta,* 362 (1974) 75–82.

Kaneo, Y., Tanaka, T., Fujihara, Y., Mori, H. and Iguchi, S., Delivery of glutathione, as a dextran conjugate, into the liver. *Int. J. Pharm.,* 44 (1988) 265–267.

Kubota, Y. and Ueki, H., Determination of the isoelectric point of bovine plasma albumin by cellulose acetate paper electrophoresis. *J. Biochem.,* 64 (1968) 405–406.

Lasen, C. and Johansen, M., Macromolecular prodrugs. I. Kinetics and mechanism of hydrolysis of *o*-benzoyl dextran conjugates in aqueous buffer and in human plasma. *Int. J. Pharm.,* 27 (1985) 205–218.

Marshall, J.J. and Rabinowitz, M.K., Preparation and characterization of a dextran-trypsin conjugate. *J. Biol. Chem.,* 251 (1976) 1081–1087.

Meister, A. and Anderson, M.E., Glutathione. *Annu. Rev. Biochem.,* 52 (1983) 711–760.

Molteni, L., Dextrans as drug carriers. In Gregoriadis, G. (Ed.), *Drug Carriers in Biology and Medicine*, Academic, London, 1979, pp. 107–125.

Schanaar, R.L., Spark, T.F. and Roseman, S., Cyanogen bromide activation of polysaccharides. Effects of reaction conditions on cationic charge and ligand content. *Anal. Biochem.,* 79 (1977) 513–525.

Wendel, A. and Jaeshke, H., Drug-induced lipid peroxidation in mice. III. Glutathione content of liver, kidney and spleen after intravenous administration of free and liposomally entrapped glutathione. *Biochem. Pharmacol.,* 31 (1982) 3607–3611.

Yamaoka, K., Tanigawara, Y., Nakagawa, T. and Uno, T., A pharmacokinetic analysis program (MULTI) for microcomputer. *J. Pharm. Dyn.,* 4 (1981) 879–885.