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7-GLYCIDOXYCOUMARIN (GOC): A FLUOROPHOTOMETRIC EPOXIDE SUBSTRATE FOR THE ASSAY OF GLUTATHIONE *S*-TRANSFERASE ACTIVITY

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Abstract—7-Glycidoxycoumarin (GOC), a new fluorophotometric epoxide substrate for glutathione *S*-transferase (GSH TFase), was conjugated regiospecifically with GSH at pH 6.5 in rat liver cytosol to yield *S*-(2-hydroxy-3-(7′-coumaroxy)-1-propyl)glutathione which was isolated by HPLC and identified with an authentic specimen by ^{13}C NMR spectroscopy. The conjugation product formed in the incubation media consisting of GOC, GSH, and 9000 *g* supernatant fractions from various tissues of the rat, was directly determined by photometry of fluorescence emission at 388 nm at an excitation wavelength of 328 nm after removal of the unreacted substrate and its enzymic hydrolysis product, 7-(1′,2′-dihydroxy-3′-propoxy)coumarin, by simple extraction with isobutyl alcohol in the presence of a saturating amount of sodium chloride. Stability of GOC at pH 6.5 markedly retarded its autoconjugation with GSH and made the fluorophotometric method sensitive enough to assay small GSH TFase activities in gel column chromatographic fractions as well as in various tissues of the animals. Apparent K_m and V_{max} for GOC in rat liver cytosol were 55 μM and 7.41 nmole/mg protein/min, respectively. GSH conjugation of GOC was catalyzed by at least two isozymes, E and AA, of hepatic GSH TFases.

Glutathione *S*-transferase (GSH TFase) plays an important role together with epoxide hydrolase in animal and human tissues in detoxifying epoxides formed as active metabolites from a wide variety of carcinogenic or toxic aromatic hydrocarbons and olefins [1]. Recent studies on GSH TFase isozymes have been carried out in most cases by using chloronitrobenzenes [2, 3] and demonstrated from gel-chromatographic profiles as well as from immunological evidence for oligomorphism of the enzyme subunits that there are at least seven isozymes in rat liver cytosol [2, 3]. GSH TFase activities for epoxides are likely to be limited in a few to several isozymes among them.

Higher epoxide-GSH conjugating activities in animal tissues and gel-chromatographic fractions have been spectrophotometrically assayed by *p*-nitrophenoxypropene oxide [4]. Rapid and convenient assays based on radioactive epoxides of olefins and arenes have been developed in which unreacted substrate is simply extracted from incubation medium into organic solvent, and an aliquot of the aqueous phase containing the water-soluble conjugate is counted. Although this approach is severely limited by availability of labeled substrates, assays based on [^{14}C]styrene 7,8-oxide [5, 6], [^{3}H]styrene 7,8-oxide [7, 8], and several [^{3}H]arene oxides [6, 8–11] have been reported.

The radiometric method, however, has met difficulty in practical use because of volatility of styrene 7,8-oxide and unstability of the arene oxides at weakly acidic to neutral pH at which their non-enzymic conjugations with GSH can be retarded at a small rate. [^{3}H]Benzo[*a*]pyrene 4,5-oxide (BPO) has been most investigated as the substrate for determining GSH TFase activity among the arene oxides proposed. However, even the well-established method of Nemoto and Gelboin employs a radioactivity scanner for determining the BPO–GSH conjugate in silica gel thin-layer chromatograms in order to separate it from BPO-bound protein after extraction of the unreacted BPO with an organic solvent [10]. As have been pointed out by Oesch *et al.* [12], most of these arene oxide methods present several disadvantages and limitations among them. The simplest method proposed by these workers [12] uses trichloroacetic acid to directly count aqueous medium containing the radioactive BPO–GSH conjugate following precipitation of BPO-bound proteins after extraction of the unreacted substrate at pH 10. However, this method has to count much non-enzymic conjugate as blank control, corresponding up to 40% of the net enzymic reaction even in liver cytosol which has the highest BPO conjugating activity of all the animal tissues.

Therefore, a highly sensitive epoxide is required for the substrate which can be readily synthesised unlike arene oxides, has an adequate solubility in water and is stable enough at weakly acidic to neutral pH in an aqueous medium to assay small GSH TFase activities. The present study provides a fluorophotometric method for permitting rapid assay of small epoxide-GSH conjugating activities in various tissues and gel-chromatographic fractions and also provides evidence for the mode of the GSH con-

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jugation reaction of the fluorophotometric substrate, 7-glycidoxycoumarin (GOC).

MATERIALS AND METHODS

Materials. GSH, a preparation free from its oxidised form, was donated from Yamanouchi Pharmaceutical Co. Ltd., Tokyo. 7-Hydroxycoumarin, 7-ethoxycoumarin, DCNB, CDNB, and epibromohydrin were purchased from Wako Pure Chemicals Co. Ltd., Osaka. Other reagents used were all reagent grade.

Synthesis of 7-glycidoxycoumarin (GOC), its GSH conjugate and hydrolysis product (DHC). To a solution of 7-hydroxycoumarin (6.17 mmole) in dry acetone (50 ml) containing finely powdered dry sodium carbonate (6.81 mmole) was added epibromohydrin (9.27 mmole), and the mixture was mechanically stirred for 2 hr at room temp. From the mixture the solvent under reduced pressure was evaporated to dryness, and the residue was dissolved in a biphase mixture of benzene and water (100 and 30 ml) by shaking. The organic layer separated was washed with water (30 ml) and dried over sodium sulphate. A crystalline residue, obtained on the evaporation of the solvent *in vacuo* from the organic solution, was dissolved in benzene (10 ml) and poured onto a silica gel column (60–80 mesh, 2 × 30 cm) packed with benzene. The column was successively eluted with benzene, benzene–acetone (50:1), and benzene–acetone (25:1). Colourless needles (0.81 g) obtained on the evaporation of the solvent from the benzene–acetone (25:1) fraction were recrystallised from benzene-*n*-hexane; m.p. 105°; 60.2% yield; u.v. λ_{max}^{MeOH} nm (ε): 320 (12670), 293 (7469, shoulder), 216 (9920), and 205 (13024); IR ν_{max}^{KBr} cm^{-1}: 3070, 1710, 1604, 1349, 1275, 1238, 1120, 1020, and 830; and MS *m/z* (relative intensity): 219 (M^+ + 1, 43%), 218 (M^+, base), 162 (-C_3H_5O, 20%), and 134 (162-CO, 38%), ^{1}H NMR $\delta_{TMS}^{(CD_3)_2SO}$ ppm: 2.71–2.93 (m, 2H, $\underline{CH_2}$—CH(O)—) and $\delta_{TMS}^{(CD_3)_2CO}$ ppm: 3.23–3.41 (m, 1H, CH_2—$\underline{CH}$(O)—), 4.46 (dd, 2H, J = 3 Hz and 11 Hz, —$\underline{CH_2}$—O—), 6.18 (d, 1H, J = 9 Hz, 3-$\underline{H}$), 6.89 (d, 1H, J = 3 Hz, 8-$\underline{H}$), 6.98 (dd, 1H, J = 3 Hz and 10 Hz, 6-$\underline{H}$), 7.54 (d, 1H, J = 10 Hz, 5-$\underline{H}$), 7.84 (d, 1H, J = 9 Hz, 4-$\underline{H}$).

The GSH conjugation product, *S*-(2-hydroxy-3-(7′-coumaroxy)-1-propyl)glutathione, was synthesised as follows: to a 4% NaOH solution (20 ml) containing GSH (2 mmole) was added a solution of GOC (2 mmole) in methanol (20 ml), and the mixture was left standing for 2 hr at room temp with mechanical stirring, adjusted to pH 6 with 10% HCl, and washed three times with ether (50 ml each). A residue obtained on the evaporation of the solvent from the mixture *in vacuo* was dissolved in water (20 ml) and poured onto an Amberlite XAD-2 column (4 × 22 cm). The column was washed with water (4 bed vol.) and eluted with methanol (3 bed vol.). An amorphous solid obtained on the evaporation of the solvent *in vacuo* from the methanolic eluate was recrystallised from aqueous methanol; 89.1% yield; u.v. $\lambda_{max}^{H_2O}$ nm (ε): 322 (14640), 290 (7520, shoulder), 216 (18800), and 199 (54400); ^{13}C NMR: data are shown in Table 1.

The hydrolysis product, 7-(1′,2′-dihydroxy-3′-propoxy)coumarin (DHC), was prepared by the treatment of a methanolic solution (5 ml) of GOC (0.23 mmole) with an aqueous sulphuric acid solution (0.5 N, 7 ml) at room temp for 2 hr. The mixture was neutralised with sodium bicarbonate and extracted with ethyl acetate in the presence of a saturating amount of sodium chloride after removal of most methanol. A crystalline residue obtained on the evaporation of the solvent, washed with a small volume of water, was recrystallised from ethyl acetate and benzene; 65% yield as colourless needles, m.p. 121°; u.v. λ_{max}^{MeOH} nm (ε): 320 (14748), 298 (8183, shoulder), 216 (10610), and 205 (15742); IR ν_{max}^{KBr} cm^{-1}: 3300, 1686, 1608, 1352, 1283, 1230, and 1032; ^{1}H NMR $\delta_{max}^{CD_3OD}$ ppm: 3.71 (d, 2H, J = 6 Hz, —O$\underline{CH_2}$—CH(OH)—CH_2(OH)), 3.98 ~ 4.30 (m, 3H, —OCH_2—$\underline{CH}$(OH)—$\underline{CH_2}$(OH)), 6.29 (d, 1H, J = 9 Hz, 3-$\underline{H}$), 6.98 (d, 1H, J = 3 Hz, 8-$\underline{H}$), 7.05 (dd, 1H, J = 3 Hz, J = 10 Hz, 6-$\underline{H}$), 7.61 (d, 1H, J = 10 Hz, 5-$\underline{H}$), 7.94 (d, 1H, J = 9 Hz, 4-$\underline{H}$); and MS *m/z* (relative intensity): 237 (M^+ + 1, 7%), 236 (M^+, 38%), 162 (-$C_3H_7O_2$, 78%), and 134 (162-CO, base).

Subcellular preparations. Male Sprague–Dawley rats weighing 260–280 g were sacrificed, and their tissues homogenized in 2 vol cold isotonic KCl solutions and centrifuged at 9,000 *g* for 20 min. The 9000 *g* supernatant fractions were adjusted with the cold KCl solution, so that 1 ml of each fraction might contain appropriate amount of the enzyme activity. A hepatic soluble supernatant fraction was separated at 105000 *g* from the post-mitochondrial supernatant fraction and dialysed against 2500 vol of isotonic KCl at 0–2° for 24 hr. The volume of the soluble supernatant fraction was adjusted with the KCl solution, so that 1 ml of the solution might correspond to 1 g of the liver or 68 mg of protein. Proteins were determined by the method of Lowry *et al.* [13].

Enzyme assay. For the assay of GSH TFase activities, the diluted 9000 *g* supernatant fraction (1 ml) was incubated at 37° for 20 min with GSH (4 mM) and a 0.25 mM GOC solution (2 ml) in a final vol of 5 ml of 0.1 M Na_2HPO_4–KH_2PO_4 buffer, pH 6.5. The substrate solution was prepared by diluting a solution of GOC (5.45 mg) in methanol (20 ml), containing 0.8% Tween 80, with the pH 6.5 phosphate buffer (80 ml). The reaction was terminated by adding sodium chloride (2 g) and immediately agitating with 2 vol of isobutyl alcohol saturated with the pH 6.5 buffer containing a saturating amount of sodium chloride. The mixture was then mechanically shaken for 10 min and centrifuged at 2000 rpm for 10 min. The fluorescence emission at 388 nm from the conjugate remaining in the separated aqueous phase was determined at an excitation wavelength of 328 nm after it was once washed with 2 vol of the NaCl-buffer-saturated isobutyl alcohol in the same manner. A blank control run was carried out under the same conditions as mentioned above by using isotonic KCl (1 ml) instead of the subcellular preparation. The rate of the non-enzymic conjugation of GOC was 2.02 nmole/ml

incubation mixture/20 min, which corresponded to 2.02% of GOC used as the substrate.

A calibration curve for the emission intensity vs concentrations of the GOC–GSH conjugate was prepared with the solutions of various amounts of the synthetic conjugate (0.1–10 nmole/ml) in the pH 6.5 buffer saturated with isobutyl alcohol and sodium chloride.

For convenience, the commercially available coumarin, 7-ethoxycoumarin, which was added as 50 μl ethanolic solutions, can be used instead of the GOC–GSH conjugate, unless available, for preparing the calibration curve. In this case, it should be noticed that the fluorescence emission intensity at 388 nm from 7-ethoxycoumarin at an excitation wavelength of 328 nm is 92% of that from the conjugate at the aforementioned range of concentrations in the same solution.

Separation of hepatic GSH TFases: GSH TFase isozymes in the hepatic soluble supernatant fraction were separated on a CM-Sephadex C-50 (Pharmacia Fine Chemicals AB, Uppsala) column by the method of Hayes *et al.*[14]. Enzyme activities for conjugating DCNB and CDNB were determined by the previous method [2].

Fluorophotometry and spectroscopy. A Hitachi Model MPF-4 spectrophotometer was used for fluorophotometry and recording of fluoresence emission, excitation, and u.v. absorption spectra. IR spectra were recorded by the KBr pellet method on a Hitachi Model EPI-G spectrophotometer, mass spectra on a Hitachi Model M-80 mass spectrometer, and ^{1}H and ^{13}C NMR spectra on a Varian Model EM-390 and a JEOL Model TX-100 NMR spectrometers, respectively.

Chromatography. HPLC was carried out on an Atto Model Constametric II equipped with an octadecylsilicone column (Nucleosil $7C_{18}$, 5 μ in particle size, 4 mm × 30 cm) and a Shimadzu Model SPD-1 stop and flow u.v. spectrophotometer. The column was eluted at a flow rate of 1 ml/min with solvent mixtures of methanol–water–acetic acid (100:200:1) for the GSH conjugate and methanol–water (6:4) for DHC. The chromatograms were obtained by monitoring at 310 nm. TLC was carried out on Merck cellulose F_{254} plates in *n*-butanol–acetic acid–water (4:1:2), and the chromatograms were visualised by ninhydrin or as fluorescence spots under a u.v. lamp (365 nm).

RESULTS

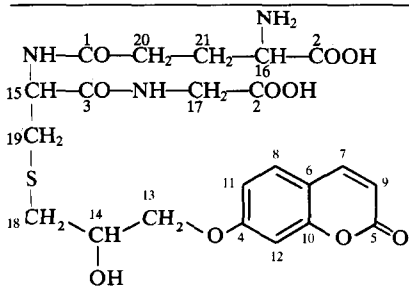
Isolation and identification of GOC–GSH conjugate formed by rat liver GSH TFase. From a large scale incubation mixture (1 l.) consisting of the rat liver soluble supernatant fraction (1.5 g protein), GOC (0.5 mmole), GSH (4 mmole), methanol (8%, v/v), Tween 80 (0.064%, w/v), and 0.1 M phosphate buffer (pH 6.5), the GSH conjugate was isolated after an hour incubation by using an Amberlite XAD-2 column (4 × 22 cm) in the same manner as had been previously reported with the regioisomeric conjugates of styrene 7,8-oxide (phenyloxiran) [15]. Prior to the application of the mixture to the resin column, the unreacted substrate and the spontaneous hydrolysis product, DHC (less than 1.7% of GOC by HPLC), were removed by the extraction with ethyl acetate, and the hepatic protein coagulated was eliminated by filtration through a diatomaceous earth bed. The conjugate eluted from the resin column by methanol showed a sharp single u.v.-absorbing peak at 10.8 min in an HPL-chromatogram obtained under the same conditions as used for the complete separation of the regioisomers of styrene 7,8-oxide-GSH conjugates at 5.6 and 6.2 min and, in a thin-layer chromatogram, also showed a single fluorescence spot at Rf 0.37 by a u.v. lamp which was also ninhydrin-positive.

The u.v. absorption, fluorescence excitation and emission spectra showed that the conjugate eluted from the HPLC column had a 7-alkoxycoumarin moiety (Fig. 1). The total structure of the conjugate was unequivocally assigned by ^{13}C NMR spectrometry as *S*-(2-hydroxy-3-(7′-coumaroxy)-1-propyl)-glutathione after it was eluted from the HPLC column. All the signals that appeared in the spectrum were well resolved as singlets before off-resonance and reasonably assigned (Table 1). The ^{13}C NMR spectrum also indicated the GOC–GSH conjugate to contain no detectable ratio of the regioisomer, *S*-(2-(7′-coumaroxy)-3-hydroxy-1-propyl)glutathione, for no ^{13}C-signals due to $—CH_2OH$ and CH–SG were found at magnetic fields of 60–76 and 35–50 ppm, respectively. Of the conjugate isolated approx. 6% were found by using a boiled liver preparation to be formed in a non-enzymic process. The non-enzymic conjugation reaction was accelerated by increasing pH of the reaction medium, it proceeded almost quantitatively at pH 10 within 20 min in the absence of the subcellular fraction. The non-enzymic conjugation reaction product (synthetic specimen) was identical with the biologically formed conjugate in all respects and also free from its regioisomer.

Profiles of enzymic conjugation of GOC in liver cytosol. The present method for enzyme assay is based on direct fluorophotometry of the GOC–GSH conjugate remaining in the aqueous phase after washing the incubation mixture with isobutyl alcohol in the presence of a saturating amount of sodium chloride to completely remove the substrate and its hydrolytic product DHC. Under these conditions, proteins which were separated from the mixture were located as a thin and fluffy disc between the organic and transparent aqueous phases after centrifugation and could be easily removed together with the organic phase by aspirating through a pipette-shaped glass-tubing. The authentic GOC–GSH conjugate (0.025–12 nmole/ml) added to the mixture remained unextracted under the aforementioned conditions and showed a linear relationship of fluorescence intensity vs concentrations. A minimum measurable concentration of the enzymically formed GSH conjugate was 0.1 nmole/ml which corresponded to 0.1% of GOC used as the substrate. The minimum measurable concentration was attained by the soluble supernatant fraction (16.3 μg protein/ml incubation mixture) equivalent to 1.0 mg wet wt of rat liver/ml incubation mixture.

Optimum pH was approx. at pH 7.5 for the enzymic conjugation of GOC in 0.1 M phosphate buffer (Fig. 2). However, the non-enzymic conjugation re-

Table 1. ^{13}C NMR spectrum of the GSH conjugate formed from GOC by enzymic or non-enzymic reaction

Structure of conjugate	No. of carbon (Chemical shift)*	
	1 (178.8 s)	12 (103.8 d)
	2 (177.7 s)	13 (73.4 t)
	3 (174.5 s)	14 (71.5 and 71.3, s)†
	4 (166.7 s)	15 (57.1 d)
	5 (164.1 s)	16 (55.9 d)
	6 (157.2 s)	17 (46.2 t)
	7 (148.3 d)	18 (37.5 t)
	8 (132.2 d)	19 (36.6 t)
	9 (116.0 d)	20 (34.4 t)
	10 (115.5 s)	21 (29.6 t)
	11 (114.3 d)	

* The spectrum was recorded with a 5% solution of the enzymically or chemically formed conjugate in deuterium oxide. Chemical shifts are expressed in ppm from TSP as an internal reference. s, d, and t represent singlet, doublet, and triplet signals after off-resonance.

† The signal was unchanged after and before off-resonance.

action occurred at a significant rate at this or higher pH; 21.7% of the GSH conjugate at pH 7.5 originated from the non-enzymic reaction. Therefore, pH 6.5 was used throughout this investigation as the most suitable pH for enzyme assay although the net enzymic conjugation rate at this pH was 68% of that at pH 7.5. K_m and V_{max} obtained from a double-reciprocal plot at the concentration ranging 0.02–0.2 mM vs the GSH conjugate formed in rat liver soluble supernatant fraction (0.8–3.2 mg protein) were 55 μM and 7.41 nmole/mg protein/min, respectively.

Of the hepatic soluble GSH TFase isozymes separated on a CM-Sephadex C-50 column by the previously reported method [14], using CDNB and DCNB as substrates only E and AA fractions had activities to catalyze the conjugation of GOC. The chromatographic profile showed that the peak area for the activity of E was about 4 times as large as that for AA so far as monitored by GOC.

Distribution of GSH TFase activities in rat tissues. GSH TFase activities were measured with 9000 *g* supernatant fractions from various tissues of male Sprague–Dawley rats in the same way as mentioned with hepatic cytosol. All the tissues examined afforded well separable and transparent aqueous layers after the extraction with isobutyl alcohol under the salting-out conditions used. No fluorescence emission was detectable in the 388 nm region from the aqueous solutions irradiated at the excitation wavelength of 328 nm when the substrate was absent. The highest specific enzyme activity was found in the liver. Next to this, testis had much higher activities than any other tissue (Table 2).

S-(2-hydroxy-3-(7'-coumaroxy)-1-propyl)glutathione was found by quantitative HPLC to be the only source for the fluorescence from the aqueous phases after incubations of GOC with the 9000 *g* supernatant fractions from all the tissues examined.

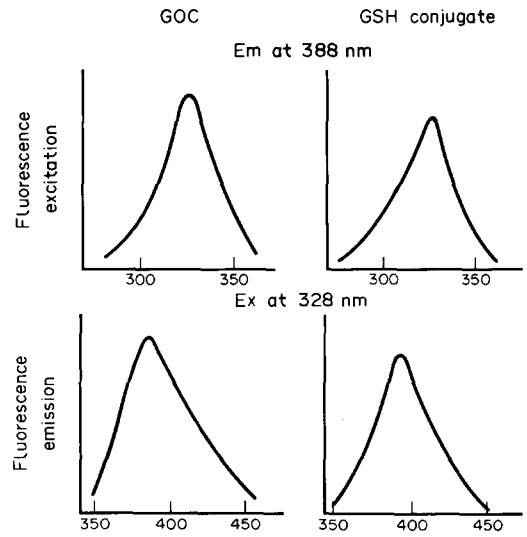


Fig. 1. Fluorescence emission and excitation spectra of GOC and its GSH conjugate. Spectra were recorded at a concentration of 0.1 μM with each sample. MeOH was used as the solvent for GOC, and 0.1 M phosphate buffer (pH 6.5) for the GSH conjugate, which was saturated with isobutyl alcohol and sodium chloride.

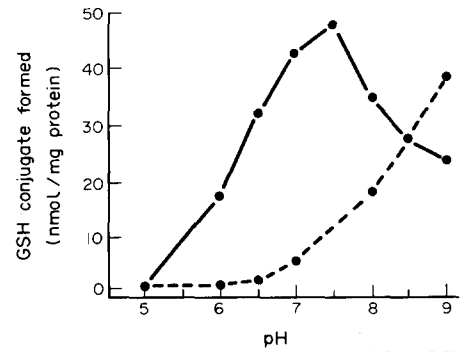


Fig. 2. An optimum pH curve for the GOC–GSH conjugation reaction mediated by GSH *S*-transferase in rat liver cytosol. 0.1 M phosphate buffers (KH_2PO_4–Na_2HPO_4) were used as reaction media. The solid line represents net enzyme activities, and the broken line autoconjugation reaction. The GOC–GSH conjugate formed was determined by fluorophotometry.

Table 2. Ubiquity of GSH *S*-transferase and epoxide hydrolase of rat tissue 9000 *g* supernatant fractions

Tissue*	GSH TFase activity† (relative value)	EHase activity† (relative value)
Liver[a]	3111 (100)	1224 (100)
Testis[a]	1524 (49)	207 (17)
Kidney[b]	952 (30)	541 (44)
Stomach[c]	538 (17)	49 (4)
Spleen[a]	406 (13)	27 (2)
Skin[c]	352 (11)	2 (0.2)
Lung[b]	316 (10)	197 (16)
Small intestine[c]	296 (9)	110 (9)
Heart[b]	196 (6)	6 (0.5)
Muscle[c]	141 (4)	21 (2)

* The organs were taken from male Sprague–Dawley rats (260–280 g) and homogenized with a Teflon-pestled Potter–Elvehjem type homogenizer (*a*), a Waring blender with steel blades (*b*), or a Polytron homogenizer (Kinematica, Switzerland) (*c*). Homogenates prepared with *b* and *c* were re-homogenized with *a*.

† Enzyme activities are expressed as pmoles GOC–GSH conjugate or DHC formed/mg protein/min. EHase: epoxide hydrolase

The isobutyl alcohol extraction of the incubation mixtures completely eliminated not only the substrate but also its hydrolysis product, DHC, that was enzymically formed during incubations. DHC extracted into isobutyl alcohol was quantified by HPLC on the same column as used for the isolation of the GOC–GSH conjugate: the glycol appeared at 6.2 min in aqueous methanol as a single u.v.-absorbing peak. DHC was formed at a much higher rate in liver than in any other tissue (Table 2). The ratio of the hepatic rate of DHC formation to that of the GSH conjugation was 1/2.54 at pH 6.5 in the presence of GSH under the same incubation conditions. DHC eluted from the HPLC column was identified by mass, u.v. absorption and fluorescence spectrometry with the authentic specimen.

Non-enzymic formation of DHC was found to be negligibly low during 20 min incubations; the glycol formed was 1.7% of GOC, at pH 6.5 when GOC was incubated with boiled tissue preparations.

DISCUSSION

GOC was found suitable as an epoxide substrate for the assay of slight GSH TFase activities in various tissues and gel column-chromatographic fractions in view of fluorescence strength, stability in an aqueous solution at pH 6.5 with and without GSH, reactivity with GSH by the catalytic action of the TFases, and simplicity in preparation and in the enzyme assay procedure. Workers, including the authors, who have tried to determine GSH conjugating activities by the previously reported methods have met a great difficulty in dealing with the unstable substrates, [^{3}H]styrene 7,8-oxide [7, 8] and [^{3}H]BPO [6, 9–11]; the former is a volatile liquid easy to polymerise, and the latter not only readily rearranges into phenols at weakly acidic to neutral pH, but also shows a maximal enzymic conjugation rate around pH 10 which makes it inevitable to cause the non-enzymic conjugation with GSH at a significant rate. Phenanthrene 9,10-epoxide has been suggested by Bend *et al.* [6] to be the most recommendable substrate of the previously introduced [^{3}H]arene oxides.

GOC yields a single GSH conjugation product, *S*-(2-hydroxy-3-(7′-coumaroxy)-1-propyl)glutathione, by GSH TFases while styrene 7,8-oxide [15] and BPO [16] are converted into two regio- and at least three separable regiodiastereoisomeric GSH conjugates, respectively, in rat liver cytosol. Styrene 7,8-oxide has very recently provided the first instance for the elucidation of the total structures of the regioisomeric GSH conjugates by ^{13}C NMR spectroscopy [15], and GOC, to our knowledge, the second instance. A partial ^{13}C NMR spectroscopic study has very recently been also carried out on estimating regioselectivity of enantiomeric K-region epoxides of arenes and azaphenanthrenes [17].

The non-enzymically formed GOC–GSH conjugate was identical with the biologically formed conjugate and completely free from the regioisomer. The rate of non-enzymic GSH conjugation of GOC significantly increased by increasing pH of the incubation medium at higher than 7.0. Therefore, pH 6.5 is recommended for retarding the non-enzymic conjugation of GOC with GSH in an aqueous medium. GOC is considerably stable at pH 6.5 in 0.1 M phosphate buffer unlike arene oxides and aryloxirans such as BPO [12] and styrene 7,8-oxide [18], so that it may be hydrolysed to the glycol, DHC, only at a rate of less than 2%/20 min at 37°.

A preliminary experiment carried out by using rat liver microsomes, indicated that microsomal epoxide hydrolase hydrolysed GOC at pH 6.5 at a rate of 60% of the maximal rate exerted at optimum pH 8.5. At pH 6.5, GOC was converted to DHC at a smaller rate than to the GSH conjugate in a rat liver homogenate or its 9000 *g* supernatant fraction: in the presence of GSH (4 mM), the rate for enzymic conversion of GOC to DHC was 40% of that of GSH conjugation. Only GOC and DHC were detected by HPLC as u.v.-absorbing materials and by TLC as fluorescence spots from the isobutyl alcohol extracts of the incubation mixtures containing 9000 *g* super-

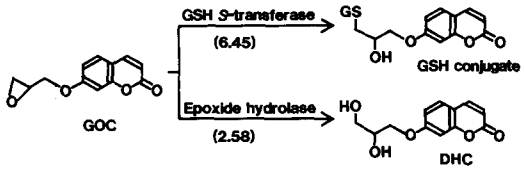


Fig. 3. Glutathione *S*-conjugation and hydrolysis of 7-glycidoxycoumarin (GOC) by post-mitochondrial fractions from various tissues of rats. Numerals in brackets represent apparent rate constants at pH 6.5 in nmole/mg hepatic protein/min.

natant fractions of various tissue homogenates from the animals.

Of GSH TFase isozymes, only E and AA, named by Jakoby *et al.* [19] had activities catalyzing the conjugation of GOC with GSH in the total activity ratio 4:1. However, none of the other isozyme fractions catalyzing the conjugations of CDNB and DCNB showed any activity towards GOC. Although it has been demonstrated by using various olefin oxides, including *p*-nitrophenoxypropene oxide, that only E [4] or both E and A fractions [19] have GSH conjugating activities, the GOC–GSH TFase activities were not found in the A fraction. GOC is likely to have an affinity to GSH TFase isozymes which differs from BPO since the latter epoxide has been demonstrated to be conjugated with GSH by the E, AA, and A fraction enzymes in the activity ratio 51:8:100 [10].

GOC was also a useful substrate for determining the conjugating enzyme activities in a variety of tissues of the animals. It is of interest that testis shows a relatively high specific activity, next to the activity of liver and higher than kidney. Existence of the high conjugating activity in testis has been demonstrated in the rat by Bend *et al.* [6] with the epoxy substrate, BP 11,12-oxide, but not with BPO, styrene 7,8-oxide and octene 1,2-oxide, all of which are conjugated at higher rates in kidney than in testis and at the highest rates in liver.

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