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PURIFICATION AND PROPERTIES OF γ-GLUTAMYL TRANSFERASE FROM NORMAL RAT LIVER

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Summary

γ-Glutamyl transferase ((5-glutamyl)-peptide: amino-acid 5-glutamyltransferase, EC 2.3.2.2) has been partially purified from both whole rat liver (600-fold) and from isolated biliary tract (1200-fold). The most highly purified fraction gave two protein bands on polyacrylamide gel electrophoresis, the major band alone having enzyme activity. The enzyme purified from biliary tract appears identical to that from whole liver preparation according to molecular weight, kinetic parameters and the effects of various inhibitors.

Three liver cell-types; parenchymal, Kupffer and biliary tract were isolated by perfusion of the rat liver in situ with collagenase, followed by selective cell isolation. Approx. 80—90% of the total recovered enzyme activity was found in the biliary tract. Nearly 50% of the apparent enzyme activity in the parenchymal cell was attributable to a nonspecific hydrolase.

Introduction

γ-Glutamyl transferase ((5-glutamyl)-peptide: amino-acid 5-glutamyltransferase, EC 2.3.2.2) is a plasma-membrane bound enzyme [1] which catalyses the reversible transfer of γ-glutamyl groups from γ-glutamyl donors to amino acids or dipeptides, or to water [2]. It has been suggested that the enzyme plays a role in amino acid transport [3]. However, recent evidence suggests that in conjunction with cysteinyl-glycine dipeptidase, γ-glutamyl transferase is involved in the reclamation of circulating glutathione [4].

The appearance of elevated activity of γ-glutamyl transferase in the serum is a useful diagnostic index for hepatobiliary dysfunctions [5]. Elevated tissue levels are closely associated with carcinogenesis in both experimental [6] and

human [7] hepatoma. The activity of the enzyme increases up to a 100-fold in rat liver after induction of hepatoma by aflatoxin [8] or diazobenzene [9].

An investigation of γ-glutamyl transferase from normal rat liver is, therefore, a prerequisite to a study of this enzyme in the diseased state. The enzyme has not previously been purified from normal rat liver probably because of the extremely low levels of activity compared to other tissues. The recent introduction of a highly sensitive fluorigenic substrate [10] makes the purification of the enzyme from normal liver now a feasible project.

This paper describes the purification of γ-glutamyl transferase from whole liver and from biliary tract, the latter accounting for 80—90% of the total enzyme activity. The two enzyme preparations were characterized, and their properties compared.

Materials and Methods

Materials

Male Sprague-Dawley rats, 6—7 weeks old, weighing 200—250 g were used. L-γ-Glutamyl-7-amino-4-methyl-coumarin was purchased from Uniscience Cambridge (Bachem Fine Chemicals). Glycylglycine, Ammediol buffer (2-amino-2-methyl-1,3 propandiol), sodium 7-deoxycholate, Lubrol WX, α-methyl-D-mannoside, collagenase Type I, pronase Type VI and gel electrophoresis reagents were of Analar grade, obtained from Sigma Chemical Co Ltd. (Poole, Dorset). $Na^{125}I$ was a product from Radiochemical Centre, Amersham. DEAE-Sepharose CL-6B, Con A-Sepharose 4B, Sephadex G-25, Sephadex G-200, Sepharose 6B and high Molecular Weight-Calibration Kit, were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Sodium pentobarbitone (Sagatal) was from May and Baker Ltd. (Dagenham, U.K.). Heparin Injection BP (5000 I.U./ml) was from Paines and Byrne Ltd. (Greenford, U.K.). All other reagents were of Analar grade, purchased from BDH, Poole, Dorset, U.K.

Methods

Protein estimation. Protein was determined by a modification of the biuret method [11] with bovine serum albumin as standard. Very low quantities of protein were measured by the fluorescamine assay [12].

Enzyme assay. γ-Glutamyl transferase activity was measured fluorimetrically with L-γ-glutamyl-7-amino-4-methyl-coumarin as substrate according to Smith et al. [10]. 1 unit of enzyme activity is defined as the amount of enzyme that converts 1 μmol substrate per min at 37°C. The hydrolytic activity was estimated in the same manner except that glycylglycine was omitted from the assay.

Analytical gel electrophoresis. Electrophoresis in duplicate was performed at 4°C on 7.5% polyacrylamide gels, in Tris-glycine buffer [13] in the presence of 0.1% Triton X-100. One of the gels was cut into 3-mm slices, and eluted overnight with 0.3 M sucrose/1 mM Na_2EDTA, pH 7.4/2% Triton X-100. The enzyme activity in each slice was then assayed. Initially, Coomassie brilliant blue R250 was used to stain for protein in the duplicate gel [14]. However, during the later purification steps, no protein bands were visible with Coomassie blue. Therefore, the enzyme preparation was radioactively labelled with

$Na^{125}I$ before electrophoresis. Radioiodination of 10 μg enzyme protein was effected by a modification of the Hunter and Greenwood procedure [15]. An aliquot of 25 μl enzyme solution was mixed with 25 μl 0.4 M sodium phosphate buffer (pH 7.4) and 5 μCi (5 μl) of a carrier-free solution of $Na^{125}I$. The following reagents (all in phosphate buffer) were added successively, with stirring: 5 μl 17.6 mM Chloramine T, 10 s later, 20 μl 12.6 mM $Na_2S_2O_5$ and then 50 μl 60.2 mM KI. After electrophoresis, the gel was immediately sliced and the protein bands were detected by counting the radioactivity in each slice. The same gel slices were eluted, and the enzyme activity assayed.

Molecular weight determination. Molecular weight estimation was performed by gel filtration in the presence of 0.1 M phosphate buffer (pH 8.0) containing 0.02% Triton X-100, on a Sepharose 6B column (2.2 × 85 cm), previously calibrated against high-molecular-weight globular protein markers. The enzyme was dialyzed overnight against the same buffer before loading onto the column. Protein was monitored at 280 nm using an LKB 2138 Uvicord S.

Isolation of different cell-types. Rats were anaesthetized by intra-peritoneal administration of sodium pentobarbitone (50 mg/kg). Perfusion of the liver in situ was carried out for 1 h at 37°C with 0.05% collagenase, using a modification of the method of Berry and Friend [16]. Parenchymal cells, Kupffer cells and biliary tract were separated according to Wootton et al. [17]. Light microscopic examination of the preparations showed that contamination by other cell types was less than 5%.

Results

Purification procedure. All the procedures were carried out at 0—4°C unless otherwise stated. The following buffers were used: buffer A, 80 mM $MgCl_2$/0.75 mM NaOH; buffer B, 0.1 M Tris-HCl (pH 8.0); buffer C, 0.5 M Tris-HCl (pH 9.0)/0.5 M NaCl/0.1% deoxycholate; buffer D, 0.2 M Tris-HCl (pH 8.0)/0.5 M NaCl/5 g/l Triton X-100/1 mM $CaCl_2$/1 mM $MgCl_2$; buffer E, 0.1 M phosphate (pH 8.0)/0.5% Triton X-100.

Whole liver

Homogenization and extraction. Liver (100 g) was homogenized in a Waring blendor with 300 ml buffer A for 2 min at maximum speed. The homogenizer was placed in ice every 15 s to minimise heat denaturation. The homogenate was centrifuged at 40 000 × *g* for 2 h in an 8 × 50 angle head rotor in an MSE Hi Spin Centrifuge. The pellet was re-homogenized for 1 min in the Waring blendor with 200 ml buffer B containing 0.5% each of deoxycholate and Lubrol. This suspension was stirred overnight and then centrifuged at 40 000 × *g* for 4 h.

Acetone precipitation. An equal volume of acetone (precooled to −15°C) was added dropwise with stirring to the detergent extract over a period of 30 min. After centrifugation for 40 min at 17 500 × *g*, the precipitate was resuspended in 100 ml buffer B containing 0.5% deoxycholate and stirred overnight. The pellet obtained from centrifugation at 40 000 × *g* for 1 h was re-extracted for 2 h in 50 ml of the same buffer and centrifuged at 40 000 × *g* for 1 h.

Butanol treatment. The combined supernatants were stirred on ice and an

equal volume of butanol (precooled to −15°C) was added dropwise over 30 min, and centrifuged at 40 000 × *g* for 30 min. The upper butanol phase was removed and precipitates discarded. Deoxycholate was added to the aqueous phase to a final concentration of 0.2%.

$(NH_4)_2SO_4$ precipitation. The aqueous solution was adjusted to 20% saturation with $(NH_4)_2SO_4$ and centrifuged at 17 500 × *g* for 30 min and the pellet discarded. The active enzyme was then precipitated from the supernatant by further addition of $(NH_4)_2SO_4$ to 80% saturation. This suspension was centrifuged as before. The active protein which floated on the surface of the solution was recovered and dissolved in buffer B containing 0.2% deoxycholate.

Desalting. A column (2 × 75 cm) of Sephadex G-25 was equilibrated with buffer B containing 0.1% deoxycholate. The enzyme solution was desalted on the column with the same buffer, at flow rate of 1 ml/min. The active fractions were pooled for subsequent ion-exchange chromatography.

DEAE-Sepharose CL-6B. The enzyme solution was applied to a column (4.5 × 15 cm) of DEAE-Sepharose CL-6B which had been equilibrated with 4 column vol. buffer B containing 0.1% deoxycholate and was washed with 2 sample vol. of the same buffer at 1 ml/min. Elution of the enzymes was carried out with a linear gradient consisting of 500 ml buffer B containing 0.1% deoxycholate and 500 ml buffer C. The enzyme was precipitated from the active fractions by 80% saturation with $(NH_4)_2SO_4$ and redissolved in buffer D. Desalting was carried out as before on a Sephadex G-25 column.

Con A-Sepharose. Affinity chromatography was carried out on a column (1.5 × 15 cm) of Con A-Sepharose 4B. The resin was equilibrated with buffer D

TABLE I

PURIFICATION OF γ-GLUTAMYL TRANSFERASE FROM WHOLE RAT LIVER

	Volume (ml)	Enzyme Activity		Protein		Specific Activity	Yield (%)	Purification
		(mU/ml)	(total mU)	(mg/ml)	(total mg)	(mU/mg protein)		(-fold)
Homogenization	375.0	27.4	10 300	79.20	29 700	0.35	100	1
Detergent extraction	235.0	38.1	8950	18.30	4300	2.08	87	6
Acetone precipitation	75.0	89.0	6680	21.60	1620	4.12	65	12
Butanol treatment	51.0	71.0	3620	6.90	352	10.30	35	29
$(NH_4)_2SO_4$ precipitation	5.0	596.0	2980	39.90	200	14.90	29	43
DEAE-Sepharose	20.5	52.8	1080	1.89	39	27.90	10	79
Con A-Sepharose	4.0	69.3	277	0.33	1	210.00	3	600
Sephadex G-200	3.5	34.9	122	0.16	0.56	218.00	1	623

TABLE II

PURIFICATION OF γ-GLUTAMYL TRANSFERASE FROM BILIARY TRACT

Three fractions of isolated biliary tracts were combined for purification of γ-glutamyl transferase.

	Volume (ml)	Enzyme Activity		Protein		Specific Activity (mU/mg protein)	Yield (%)	Purification (-fold)
		(mU/ml)	(total mU)	(mg/ml)	(total mg)			
Whole liver homogenate	75	18.0	1350	52.9	3970	0.34	100	1
Isolated biliary tract	22	38.5	847	3.18	70	12.11	63	36
Detergent extraction	21.2	32.0	678	0.91	15.9	35.2	50	104
$(NH_4)_2SO_4$ precipitation	6.0	93.0	560	0.43	2.6	219	41	644
Con A-Sepharose	1.75	132.0	231	0.33	0.6	398	17	1170

at a flow rate of 1 ml/min. The enzyme solution was applied and the column washed with 200 ml of the equilibrating buffer. The enzyme was eluted with the same buffer containing 0.25 M α-methyl mannoside. The active fractions were concentrated and desalted as before.

Sephadex G-200. The enzyme was then subjected to gel filtration by ascending chromatography on a column (1.5 × 60 cm) of Sephadex G-200 using buffer E, at a flow rate of 0.05 ml/min. The active fractions were pooled and concentrated by vacuum dialysis.

Table I summarises the steps in a typical isolation procedure of γ-glutamyl transferase from 100 g liver. There was marked loss of enzyme activity but a 600-fold purification was achieved.

Biliary tract

Biliary tracts were isolated by collagenase perfusion, which served as an important purification step. The biliary tracts were homogenized for 1 min at low speed with a Polytron (Kinematica GMBH) and for 1 min with a motor-driven Teflon Potter homogenizer. Extraction of the enzyme from the plasma membrane, $(NH_4)_2SO_4$ precipitation and affinity chromatography on Con A-Sepharose were carried out exactly as before. Although the organic solvent, ion-exchange and gel filtration chromatography steps were omitted, a final enrichment of 1200-fold was achieved and there was substantially higher yield of enzyme activity. A summary of purification of γ-glutamyl transferase from three biliary tract preparations is presented in Table II.

Enzyme characterization

Polyacrylamide gel electrophoresis. The whole liver enzyme preparation was found to be heterogeneous when analysed on polyacrylamide gels (Fig. 1). At least four bands of protein were visible after prolonged staining with Coomassie blue. Assay of enzyme activity in the gel slices showed that a clear separation was not achieved. Although the broad spread of activity along the gel indicates

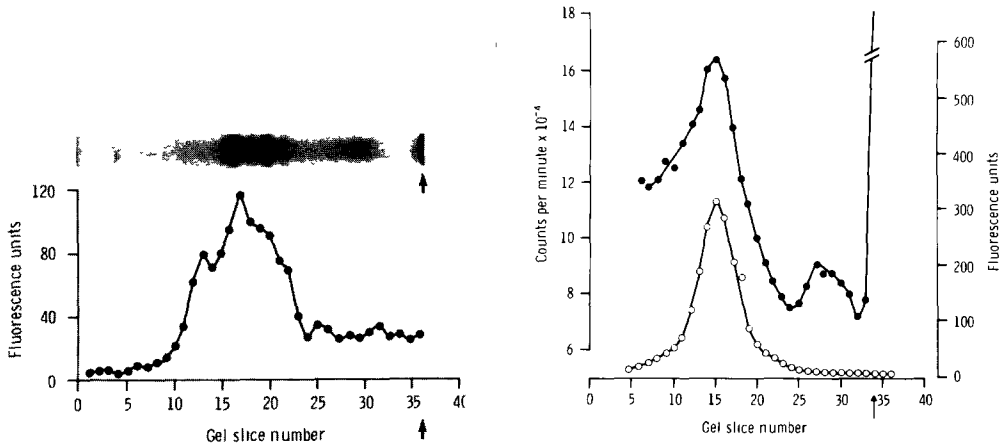

Fig. 1. Polyacrylamide gel electrophoresis of γ-glutamyl transferase from whole liver. Arrows indicate position of marker dye (Bromophenol blue). Electrophoresis of 34 μg protein in 7.5% acrylamide gel was for 6.5 h at constant voltage and 3 mA/gel. γ-Glutamyl transferase activity was assayed (●——●) in the 3-mm gel slices.

Fig. 2. Polyacrylamide gel electrophoresis of γ-glutamyl transferase from biliary tract. Arrow indicates position of marker dye plus free iodide. Electrophoresis of 10 μg protein in 7.5% acrylamide gel was for 7 h at constant voltage and 3 mA/gel. ^{125}I (●——●) and γ-glutamyl transferase activity (○——○) were estimated in the 3-mm gel slices.

the presence of more than one component, the peaks do not correspond directly to the protein bands stained with Coomassie blue. However, the radioiodinated protein from biliary tract showed a discrete peak of enzyme activity which corresponds to the major protein peak (Fig. 2). There was only one other minor protein contaminant towards the lower part of the gel. If a homogeneous enzyme is required, preparative gel electrophoresis could be carried out to remove this contaminant.

Molecular weight determinations. Determination of the apparent molecular weight was carried out as described in Methods. The K_{av} was estimated for each standard protein (thyroglobulin, catalase and α-amylase) using the relationship $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e, V_0, and V_t are the elution volume of the

TABLE III

COMPARISON OF PROPERTIES BETWEEN γ-GLUTAMYL TRANSFERASE FROM WHOLE LIVER AND BILIARY TRACT

The kinetic parameters were calculated from direct linear plot of the data [26]. Non-parametric 95% confidence limits [27] on the kinetic parameters are shown in parentheses. GGT, γ-glutamyl transferase; GGH, γ-glutamyl hydrolase.

	Whole liver	Biliary tract
Concanavalin A binding (%)	93	95
Molecular weight (apparent)	250 000	250 000
K_m^{app} (mM)	0.39 (0.32—0.49)	0.49 (0.37—0.65)
V^{app} (nmol $min^{-1} \cdot mg^{-1}$ protein)	723 (634—835)	994 (788—1250)
GGH/GGT (%)	7.6	6.5
$ZnCl_2$ inhibition (50%)	0.40 mM	0.40 mM

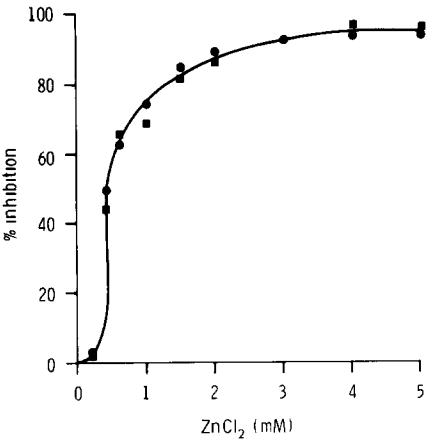

Fig. 3. Effect of $ZnCl_2$ on the enzyme preparations (whole liver, ●——●; biliary tract, ■——■). Enzyme activity was measured with the standard assay in the presence of the indicated concentration of $ZnCl_2$.

given protein, the void volume and total volume of gel bed, respectively. A calibration curve was plotted with K_{av} against the corresponding log molecular weights. An apparent molecular weight of 250 000 was obtained for γ-glutamyl transferase from both the whole liver and biliary tract.

Kinetic parameters. Estimates of the apparent K_m and V for L-γ-glutamyl-7-amino-4-methyl-coumarin were obtained from variation of the initial rate reaction at a range of donor substrate concentrations (0.07—0.36 mM L-γ-glutamyl-7-amino-4-methyl-coumarin) and fixed acceptor (15.5 mM glycylglycine) concentration. The results are shown in Table III.

Hydrolytic activity of the enzyme from whole liver and biliary tract was 7.6 and 6.5% of the respective transferase activities.

Effects of metal ions and inhibitors. $MgCl_2$ had no effect on either enzyme preparation. $ZnCl_2$ markedly inhibits both the enzymes, giving sigmoid curves (Fig. 3). This inhibition was reversible by 10 mM EDTA. However, alone, EDTA had no effects on the enzyme activity. The thiol-group inhibitor, *p*-chlo-

TABLE IV

DISTRIBUTION OF ENZYME ACTIVITY BETWEEN CELL TYPES

Results of mean ± S.E. of γ-glutamyl transferase in various cell types, calculated from eight separate perfusions. Recovery of enzyme activity in isolated cells was 86% of that in whole liver. GGT, γ-glutamyl transferase; GGH, γ-glutamyl hydrolase.

	γ-Glutamyl transferase			γ-Glutamyl hydrolase
	mU	Distribution (%)	Specific activity (mU/mg protein)	% Hydrolase (GGH/GGT X 100%)
Whole liver	522	100	0.33	17
Biliary tract	409	85	12.30 ± 1.9	13
Parenchymal	28	10	0.10 ± 0.02	47
Kupffer	13	5	0.37 ± 0.07	14

romercuribenzoate (0.1 mM) gave partial inhibition of 15%.

A summary of the properties of whole liver and biliary tract γ-glutamyl transferase is shown in Table III.

Distribution of enzymes in the cell-types

Table IV shows that although parenchymal cells contribute the bulk of the total cell population, they have the lowest specific activity and that 80—90% of the total enzyme activity in the whole liver is found in the biliary tract. Nearly 50% of the apparent enzyme activity in the parenchymal cells was attributable to a hydrolase. As the purified transferase expressed only 6—7% hydrolytic activity, this indicates that most of the enzyme activity in the parenchymal cell is a non-specific γ-glutamyl hydrolase.

Discussion

This paper reports the purification of γ-glutamyl transferase from normal rat liver and is a modification of the method of Huseby [18] which was developed for human liver. Several important changes were made. During the purification procedure, dialysis of the enzyme led to irreversible loss in activity. Attempts to concentrate the enzyme solution with Amicon Diaflo PM30 ultrafiltration also resulted in marked loss of activity. This problem was overcome by concentrating the large volumes of enzyme solution by 80% saturation with $(NH_4)_2SO_4$ and subsequent desalting on Sephadex G-25. The recovery of enzyme activity from this technique was high (90—95%) and a further purification occurred.

Salt gradient elution of γ-glutamyl transferase from DEAE-Sephadex as described by Huseby resulted in shrinkage of the gel giving rise to irregular and irreproducible elution patterns. However, use of DEAE-Sepharose alleviated these problems.

Although considerable purification of γ-glutamyl transferase from whole liver was obtained using these techniques, at least four protein bands were visible on gel electrophoresis, none of which exactly corresponded with γ-glutamyl transferase activity.

Investigation of the distribution of γ-glutamyl transferase activity in parenchymal, Kupffer and biliary tract cells showed that the majority of γ-glutamyl transferase activity was found in the biliary tract, in agreement with the findings of Wootton et al. [17]. In addition, it was found that at least 50% of the γ-glutamyl transferase activity expressed by parenchymal cells could be accounted for by a nonspecific hydrolase.

Purification of γ-glutamyl transferase from biliary tract gave a higher yield of enzyme, since fractionation with organic solvents could be omitted and the final preparation had a specific activity higher than that obtained using whole liver. Visualization of protein by Coomassie blue after gel electrophoresis of purified biliary tract enzyme was unsuccessful, although a sharp peak of γ-glutamyl transferase activity was found in the duplicate gels. This may be due to the carbohydrate content of the enzyme [19] or to the presence of Triton X-100, both of which can interfere with protein staining. Radioiodination of the enzyme preparation prior to gel electrophoresis showed only two proteins

to be present, the majority of the label being associated with γ-glutamyl transferase activity.

A comparison of the properties of the enzyme from whole liver and from biliary tract showed no significant difference between them. The percentage of enzyme binding to concanavalin A was similar in both cases. The 5—10% of the enzyme activity which did not bind to this lectin is probably the sialated fetal form [20].

Enzyme from both sources had an apparent molecular weight of 250 000 as measured by gel filtration. The molecular weight determinations were performed in the presence of Triton X-100 and formation of detergent micelles will almost certainly give rise to an increase in the apparent molecular weight which has also been observed in detergent-solubilized γ-glutamyl transferase obtained from rat kidney [21,22]. However, enzymic proteolysis was not used to free the enzyme from the membrane and, therefore a higher molecular weight is expected due to the presence of the hydrophobic tail than found in some other preparations of γ-glutamyl transferase [18,23].

Kinetic data were consistent with reports of γ-glutamyl transferase purified from other sources [24]. Although slight activation by Mg^{2+} was reported for hog kidney enzyme [2], no significant effect on the enzyme activity was observed in this present investigation when γ-glutamyl transferase (either whole liver, or biliary tract) was assayed in the presence of 10 mM $MgCl_2$. This observation is in agreement with reports by Szewczuk and Baranowski [25] on bovine kidney enzyme. However, $ZnCl_2$ was a potent inhibitor of both the enzyme preparations, resulting in sigmoid curves.

These results indicate no significant difference between the properties of the enzyme preparations, and therefore suggest that the whole liver γ-glutamyl transferase is essentially biliary tract γ-glutamyl transferase, since the latter contributes 80—90% of the total enzyme activity in the whole liver. The extremely low levels of activity in the parenchymal cells is further evidence to suggest that it is unlikely that γ-glutamyl transferase is involved in amino acid transport [3] in liver, an organ with a high affinity for circulating amino acids.

Owing to its broad specificity in hydrolysing glutathione S-conjugates, γ-glutamyl transferase has been implicated in metabolism of various xenobiotics [28]. However, in isolated hepatocytes the formation of GSH adducts was not followed by further breakdown of GSH moiety [29,30], despite the presence of γ-glutamyl transferase in whole liver. This observation could be explained by the restriction of γ-glutamyl transferase activity largely to the biliary tract. The extremely low level of γ-glutamyl transferase activity in the parenchymal cell population also accounts for the lack of any measurable uptake by exogenous [U-^{14}C]glycine-labelled GSH and GSSG during perfusion via the portal vein [4].

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