Biochemical Pharmacology, Vol. 32, No. 17, pp. 2529–2534, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

IDENTIFICATION AND QUANTITATION OF GLUTATHIONE IN HEPATIC PROTEIN MIXED DISULFIDES AND ITS RELATIONSHIP TO GLUTATHIONE DISULFIDE

REGINA BRIGELIUS, CHRISTIAN MUCKEL, THEODORUS P. M. AKERBOOM and HELMUT SIES
Institut für Physiologische Chemie I, Universität Düsseldorf, Düsseldorf,
Federal Republic of Germany

(*Received* 24 *November* 1982; *accepted* 8 *March* 1983)

Abstract—The amount of glutathione present in hepatic protein mixed disulfides was determined to be 20–30 nmole/g liver. This was established using two specific enzymatic methods: (a) the coupled assay with DTNB and glutathione (GSSG) reductase and (b) a newly developed test using GSH transferase and 1-chloro-2,4-dinitrobenzene for the estimation of GSH released from proteins after borohydride treatment; further, these results were confirmed by HPLC analysis. Thus, authentic glutathione makes up only 2–6% of the value for total protein mixed disulfides. The latter were determined with the generally employed *o*-phthalaldehyde assay, which is not necessarily specific for GSH. The amount of glutathione mixed disulfides depends linearly on the content of glutathione disulfide in the liver cell in the range studied. By increasing the GSSG levels from 20 to about 60 nmole/g liver with paraquat, nitrofurantoin or *t*-butyl hydroperoxide, glutathione protein mixed disulfides are increased by a similar amount.

Research into the biological significance of protein mixed disulfides has revealed regulatory potential for membrane thiols in general and enzyme protein thiols in particular [1, 2]. It has recently been shown that substantial changes in mixed disulfides occur also in conditions of toxicological interest, for instance in redox cycling elicited by paraquat as demonstrated for liver [3] and lung [4]. While it appears to be generally held that protein mixed disulfides in cells are formed with glutathione, we noted in the work on paraquat [3], as well as in a survey of existing methods [5], that usually non-enzymatic methods are employed, so that specificity for glutathione released from mixed disulfides upon chemical reduction is not guaranteed.

In the present work, we have addressed the problem of identification and quantitation of glutathione in protein mixed disulfides from rat liver. For this purpose, two enzyme reactions and HPLC analysis were applied, and the results were compared to those observed with the *o*-phthalaldehyde method. The first enzyme reaction is the coupled assay with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)* and glutathione reductase [5, 6]; the second is a newly developed assay using glutathione transferase and 1-chloro-2,4-dinitrobenzene (CDNB), similar to a recent application of the transferase employing *o*-dinitrobenzene as substrate [7].

It is of interest to note that, on a molar basis, the cellular content of authentic glutathione present in protein mixed disulfides in control livers is similar to the content of glutathione disulfide, about 20–30 nmole/g liver.

Compounds known to increase intracellular levels of oxidized glutathione, such as paraquat [3], nitrofurantoin [8] or *t*-butyl hydroperoxide [8], were used to study whether the experimental increase in GSSG levels is accompanied by a change in glutathione mixed disulfides. A significant increase in both GSSG and protein glutathione mixed disulfides after perfusion of the livers with these compounds was observed.

MATERIALS AND METHODS

Livers from male Wistar rats (220–250 g) were perfused with Krebs–Henseleit medium saturated with an oxygen–carbon dioxide mixture (95:5) [9]. Additions were made directly before the portal vein using infusion pumps, or the compound to be investigated was dissolved in the perfusion medium. Experiments were terminated by freeze-clamping the liver with aluminium tongs cooled to the temperature of liquid nitrogen. Frozen liver pieces were pulverized in a mortar under liquid nitrogen.

Sample preparation

Protein-bound mixed disulfides. Protein pellets were prepared by precipitating 500 mg liver powder with 3 ml 1 N perchloric acid and washed three times with 10 ml 1 N perchloric acid after which no free soluble GSH was detected. The pellets were reduced as described in [3] in the presence and absence of guanidinium chloride (4 M). After the reduction procedure the samples were centrifuged at 100,000 *g* for

* Abbreviations: Glutathione (reduced), GSH; glutathione disulfide, GSSG; 1-chloro-2,4-dinitrobenzene, CDNB; 5,5′-dithiobis(2-nitrobenzoic acid), DTNB.

15 min. The supernatant was neutralized and estimated for glutathione by methods 1–4 below.

Intracellular glutathione disulfide. For the determination of intracellular GSSG in liver extracts, GSH was trapped with *N*-ethyl-maleimide [5, 8]. Excess *N*-ethyl-maleimide was quantitatively removed by chromatography on QAE Sephadex as described in [8]. GSSG was measured by following the oxidation of NADPH at 340–400 nm in a Sigma ZWS II dual-wavelength spectrophotometer after addition of GSSG reductase.

Analytical procedures

1. *Fluorescence assay (o-phthalaldehyde method).* Formation of the fluorescent adduct of *o*-phthalaldehyde and GSH or other thiols was determined as described before [3, 10]. Data were expressed as GSH-equivalents obtained on the basis of fluorescence calibration with external GSH.

2. *Kinetic assay.* The procedure was performed as described in [5]. As the reaction rate of the system was influenced by traces of borohydride and/or compounds released from the proteins, each single estimation was calibrated by the addition of a known amount of GSSG (internal standard) to the cuvette.

In the presence of sample the slope obtained for the internal standard was lower than in its absence (external standard) and depended on the sample dilution. The enzyme blank was corrected by the factor internal/external standard slope and in this way identical values for each sample dilution were obtained. As the kinetic assay made no difference between reduced or oxidized glutathione, data were calculated in GSH-equivalents.

3. *GSH transferase assay.* The reaction mixture contained 500 μl 0.1 M potassium phosphate buffer, pH 7.0, 100 μl neutralized sample and 10 μl of a 10 mM CDNB solution in ethanol. The reaction was started with 5 μl enzyme solution (12 U/ml), and the formation of the *S*-(2,4-dinitrophenyl)-glutathione conjugate was monitored at 340–400 nm in a Sigma ZWS II dual wavelength spectrophotometer (Biochem. Co., München, F.R.G.), reaching the end-point 5 min after enzyme addition. In the presence of guanidinium chloride at least 15 min were required for the reaction to reach the end-point.

4. *HPLC analysis.* Compounds released by borohydride treatment from pellets of liver tissue were analysed on a Perkin–Elmer Series 2 HPLC equipped with a Waters μ-Bondapak NH_2 analytical column. The samples were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene as described by Reed *et al.* [11]. The sample (100 μl) was injected and eluted by an ammonium acetate–acetic acid–methanol gradient [11]. After an isocratic period of 10 min the linear gradient (2%/min) was started.

Chromatography on Dowex WX8

For the chromatography of compounds released from protein pellets the reduction procedure [3] was slightly modified. Pellets from 500 mg liver powder, prepared as described above, were quantitatively transferred into 1.75 ml of 0.1 M Tris buffer, pH 8.0, plus 2 ml of 8 M guanidinium chloride. 100 mg $NaBH_4$ and 50 μl *n*-octanol as antifoam were added. Then the mixture was homogenized for 20 sec in an Ultra Turrax and incubated at 40° for 30 min. For protein precipitation and removal of excess borohydride 1 ml of 6 N perchloric acid instead of 50% metaphosphoric acid was used because the perchlorate anions can be removed as potassium salt before chromatography. After centrifugation at 100,000 *g* the supernatant was brought to pH 6.2 with 6 N KOH and the precipitating potassium perchlorate removed by centrifugation. This supernatant was diluted 20 times with water to decrease the guanidinium chloride concentration and to create the conditions required for the binding on an ion exchanger.

Before applying to the column, samples were mixed with [^{3}H]GSH as a marker. An aliquot of 0.5 ml was added to a 0.6 × 8 cm Dowex WX8 column (K^+-form, 20–50 mesh) which then was eluted with 1 mM K-phosphate buffer, pH 6.2 and subsequently with 4 M guanidinium chloride, adjusted to pH 6.2 with KOH (Fig. 2). The fractions were tested for radioactivity and *o*-phthalaldehyde-reactive material.

Materials

GSH transferase was isolated from rat liver by the method of Habig *et al.* [12] (transferase A) or obtained from Sigma (München, F.R.G.) (equine liver). [^{3}H-Gly]GSH (1 Ci/mmole) was purchased from NEN (Dreieich, F.R.G.). All other biochemicals and enzymes were obtained from Merck (Darmstadt, F.R.G.) or Boehringer (Mannheim, F.R.G.) except for Dowex WX8 (Serva, Heidelberg, F.R.G.).

RESULTS

Assay of glutathione with CDNB and GSH transferase

For the estimation of glutathione released from protein pellets by borohydride, the methods usually applied either were not sensitive enough, as in the case of the glyoxalase assay, or were impaired by traces of borohydride and the excess amount of guanidinium chloride, like the DTNB coupled glutathione reductase assay (see Materials and Methods). For this reason we followed the example of Asaoka and Takahashi [7] and developed a new assay for glutathione with GSH transferase which turned out to be highly sensitive and not hampered by the difficulties mentioned above.

This test is based on the conjugate formation of 1-chloro-2,4-dinitrobenzene (CDNB) and GSH after addition of GSH transferase. The reaction product *S*-(2,4-dinitrophenyl)-glutathione shows a broad absorption maximum in the range 330–360 nm with an extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ at 334 nm [12]. By monitoring the formation of the conjugate at the wavelength pair 340–400 nm, an extinction coefficient of 6.9 $mM^{-1}cm^{-1}$ was calculated in agreement with optical spectra (not shown). The end-point of the reaction proved to be linear with the amounts of glutathione employed, ranging from 1 to 25 μM (Fig. 1) and higher. Assays 1–4 (see Materials and Methods) showed identical results both for standard GSH solution and for liver

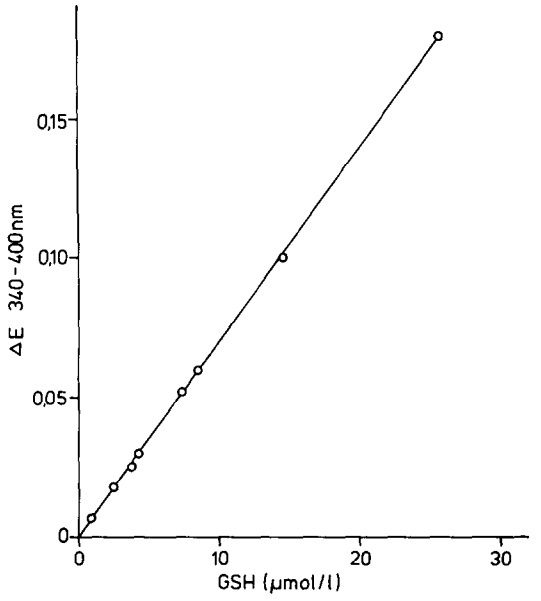

Fig. 1. Assay for GSH with GSH transferase and 1-chloro-2,4-dinitrobenzene. GSH concentration was calibrated by the glyoxalase method [5]. For details see Materials and Methods.

samples, proving the validity of each method. The new assay is capable of quantifying an amount of 0.2 nmole GSH in the cuvette (Fig. 1) corresponding to a solution of about 0.3 μM of GSH, an advantage over the assay described recently [7] where a concentration of at least 5 μM was needed.

Chromatography of compounds released from protein pellets by borohydride and guanidinium chloride treatment

Mixed disulfides are usually calculated from the difference in GSH-equivalents before and after borohydride reduction of tissues. As outlined in [3] an increase in GSH-equivalents after treatment of liver tissue with borohydride was observed only with the unspecific *o*-phthalaldehyde test but not with enzymes specific for glutathione. Mixed disulfides characterized as *o*-phthalaldehyde-reactive material were completely recovered in protein pellets [3]. For this reason, and also because the high soluble GSH concentration is not interfering, we used only protein pellets in the present work.

The material released from protein pellets by borohydride in the presence of guanidinium chloride was chromatographed on a Dowex WX8 cation exchange column. For this purpose [^{3}H]GSH (labelled in the glycine residue) was added as a marker before the samples were applied to the Dowex WX8 column.

The chromatography resulted in the separation of

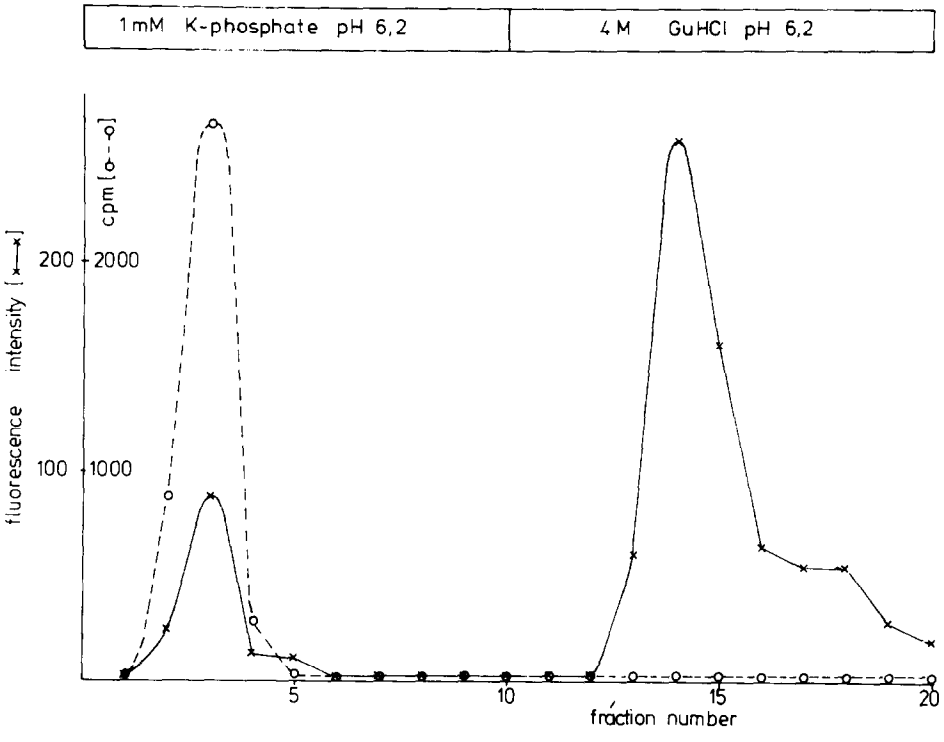

Fig. 2. Chromatography of borohydride-releasable material from the protein precipitate of rat liver. Protein pellets from frozen rat liver were reduced with borohydride + guanidinium chloride (see Materials and Methods). Excess borohydride was removed with 6 N perchloric acid and then the solution was neutralized with 6 N KOH. The potassium perchlorate was removed by centrifugation, the supernatant was diluted 20 times with water, and [^{3}H]GSH was added as a marker. An aliquot of 0.5 ml was added to a 0.6 × 8 cm Dowex 50 WX8 column (K^+-form, 20–50 mesh). Elution with K-phosphate and guanidinium chloride is indicated in the top panel.

Table 1. Protein-bound mixed disulfides (nmole/g liver) and GSSG contents (nmole/g liver) in control livers and livers perfused with compounds inducing oxidative stress

	Protein-bound mixed disulfides				
	Methods specific for GSH				
	DTNB + glutathione reductase	CDNB + GSH-transferase	HPLC	Unspecific method *o*-Phthalaldehyde	GSSG*
Control,	29 ± 2 (15)	25 ± 2 (12)	32 ± 3 (10)	452 ± 26 (14)	20 ± 0.5
20 min perfusion†	34 ± 6 (4)	30 ± 4 (5)	§	1800 ± 110 (8)‖	(6)
+guanidinium chloride‡					
t-BOOH (0.1 mM),					
30 min perfusion	94 ± 9 (3)	86 ± 16 (7)	94 ± 10 (3)	499 ± 35 (4)	110 ± 6 (7)
Nitrofurantoin (80 μM),					
60 min perfusion	54 ± 8 (5)	38 ± 3 (4)	64	505 ± 42 (5)	46 ± 2 (7)
Paraquat (1 mM),					
120 min perfusion	40 ± 3 (3)	42 ± 2 (4)	57 ± 8 (4)		40 ± 3 (5)

* GSSG was corrected for bile as given in Fig. 4.
† control values were not dependent on perfusion time.
‡ 4 M guanidinium chloride was present during the reduction procedure.
§ GSH could not be separated from guanidinium chloride eluting at the same retention time in the system used.
‖ Data taken from [3].

two peaks of *o*-phthalaldehyde-reactive material (Fig. 2). Only the first fraction not bound to Dowex contained a compound eluting like [^{3}H]GSH, amounting to 20 ± 1.7% ($n = 4$) of the *o*-phthalaldehyde-reactive material. The remaining material was eluted with 4 M guanidinium chloride in a second peak. In this fraction glutathione was not detectable either by radioactivity or by enzymatic analysis.

Quantitation of protein-bound glutathione

Specific enzymatic quantitation of glutathione in samples obtained from reduced protein pellets resulted in values of about 30 nmole/g liver, irrespective of the assay used (Table 1). This is only 2 or 6% of the amount estimated for the unspecific *o*-phthalaldehyde method depending on whether reduction was carried out with or without guanidinium chloride, respectively. This indicates that the 20% of *o*-phthalaldehyde-reactive material which contained glutathione and which was not bound to the Dowex column (Fig. 2) may also contain compounds different from glutathione. Omission of guanidinium chloride in the reduction mixture did not influence the levels determined enzymatically but yielded a much lower amount of mixed disulfides, as detected with *o*-phthalaldehyde. The low amount of glutathione in mixed disulfides is not due to a chemical modification during the reduction procedure since a 90% recovery of GSH or GSSG added to the protein pellets before borohydride treatment was obtained with all three of the assays used.

A method for separation and quantitation of low amounts of thiols in a highly reproducible way has been developed by Reed *et al.* [11]. These authors used HPLC analysis on an anion exchange column for separation of thiols from which the SH-group was blocked with iodoacetic acid and the amino group derivatized with dinitrofluorobenzene.

A typical HPLC pattern obtained after reduction of protein pellets omitting guanidinium chloride is shown in Fig. 3B. Two distinct peaks were separated on the μ-Bondapak NH_2 column. The second peak eluted at a position found for a glutathione standard (Fig. 3C). By adding the glutathione standard to the sample directly, one single peak was obtained at the corresponding retention time. For the sample shown in Fig. 3B the glutathione mixed disulfide content was calculated to be 34 nmole/g liver, which is in good agreement with the results obtained with the enzymatic methods (Table 1). Glutathione release is dependent on the presence of borohydride, because incubation without the reducing agent leads to only minute amounts of free glutathione (Fig. 3A).

The retention time of the first peak did not correspond to that of naturally-occurring thiols such as cysteine, cystine, cysteinylglycine, cystinylbisglycine, cysteamine or cystamine, or to that of glutamate. Whereas these compounds would elute at times close to that of the unknown peak ([11] and H. Menzel, personal communication), the addition of the substances mentioned above to the samples led to separate peaks. The first peak was also observed after treatment in the absence of borohydride (Fig. 3A).

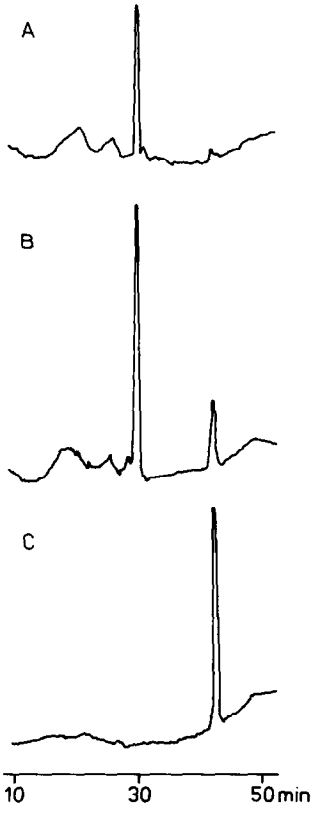

Fig. 3. HPLC analysis of borohydride-releasable material from the protein precipitate of rat liver. Protein pellets were treated in the absence of guanidinium chloride and the compounds released were derivatized and chromatographed according to [11] as given in Materials and Methods. The start of the gradient is indicated by an arrow. Chromatographic pattern of (A) compounds released from protein pellet without borohydride; (B) compounds released from protein pellet by borohydride; (C) GSH standard, corresponding to 114 nmole/g liver.

Relationship between protein glutathione mixed disulfides and GSSG

Compounds known to increase intracellular levels of GSSG, such as paraquat [3], nitrofurantoin [8] and *t*-butyl hydroperoxide [8], were employed to study the relationship between protein glutathione mixed disulfides and GSSG. The mechanisms underlying the increase in GSSG by these compounds is known to be mediated by GSH peroxidases. Paraquat and nitrofurantoin undergo redox-cycling [13] thereby continuously producing superoxide radical, which is dismutated to H_2O_2 which is, in turn, a substrate for GSH peroxidase. *t*-Butyl hydroperoxide is reduced to *t*-butanol by GSH peroxidase.

Table 1 shows the data for the contents of GSSG and glutathione mixed disulfides of livers perfused with the three compounds. As indicated by the enzymatic methods and HPLC analysis 60 and 120 min after addition of nitrofurantoin and paraquat, respectively, the increase in GSSG is nearly the same as for glutathione mixed disulfides, and also after addition of *t*-butyl hydroperoxide in the concentrations used for Fig. 4. A possible increase with the *o*-phthalaldehyde method is masked because of the experimental scatter.

DISCUSSION

Methodological aspects

The present investigation describes a new assay system for the estimation of glutathione in connection with the quantitation of the portion of glutathione in hepatic mixed disulfides. The test uses the conjugate formation of 1-chloro-2,4-dinitrobenzene and GSH in the presence of GSH transferase, which can be directly monitored spectrophotometrically. With the new test the amount of glutathione bound to proteins was found to be about 20–30 nmole/g liver wet weight and was confirmed by a comparison with the DTNB/glutathione reductase assay and with HPLC analysis. This amount was substantially lower than that obtained with the unspecific fluorometric assay using *o*-phthalaldehyde.

Furthermore, values obtained with the fluorometric assay differ largely depending on whether guanidinium chloride was present in the reduction mixture. Without guanidinium chloride a much smaller increase in the *o*-phthalaldehyde-reactive material was observed in both the whole liver tissue (unpublished results) and in protein pellets obtained therefrom (Table 1), whereas the release of glutathione was nearly unaffected by guanidinium chloride. This is in agreement with the finding by Modig [14] that the amount of glutathione released by borohydride in Ehrlich ascites tumour cells depended only slightly on the presence of urea, which was used for unfolding in the experiments. With increasing urea concentrations up to 6 M, the released GSH was raised 1.5-fold whereas non-protein SH-com-

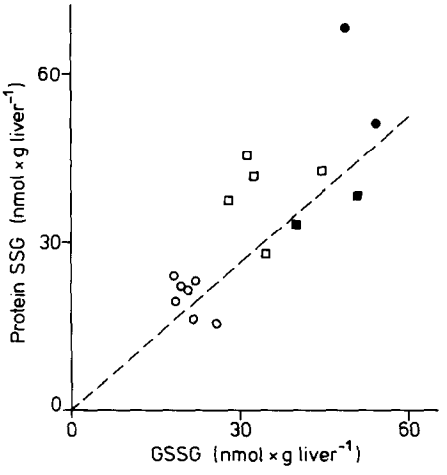

Fig. 4. Relationship between GSSG and protein-bound glutathione in liver cells. GSSG values were corrected for biliary GSSG using a volume of 2.3 µl bile per g liver [22]. Protein glutathione mixed disulfides were measured with GSH transferase (assay 3 in Materials and Methods). Control (○); paraquat, 1 mM (□); nitrofurantoin, 80 µM (■); *t*-butyl hydroperoxide, 70 µM (●). The correlation between GSSG and glutathione mixed disulfides seems to be 1:1 by the fit of the measured points to the hypothetical 45° line.

pounds were increased 6-fold [14]. In conclusion, since the release of glutathione is independent of the presence of guanidinium chloride in the reduction mixture, the latter can be omitted.

Although *o*-phthalaldehyde is useful to determine free soluble GSH in liver cells, its application for the estimation of glutathione mixed disulfides obviously leads to high values because compounds other than glutathione are also measured. The fluorescence behaviour of such compounds may differ from that of glutathione so that fluorescence may not reflect glutathione concentration. Similar comments were published by Beutler and West [15] regarding the estimation of GSSG with *o*-phthalaldehyde by the method of Hissin and Hilf [16]. They found that under conditions where GSSG reacts with *o*-phthalaldehyde only about 12% of the *o*-phthalaldehyde-reactive material from rat liver actually represents GSSG.

The nature of the *o*-phthalaldehyde-reactive material needs to be identified. In view of the balance of GSH loss vs protein mixed disulfides found in the paraquat experiments [3], and in view of the nutritional [17] and diurnal [18] variation of GSH and protein mixed disulfides adding up to a constant so-called 'total glutathione', there may be further metabolism of the glutathione moiety once bound to cellular protein sites [19]. When released by chemical reduction from the protein, such a metabolite of glutathione is neither a substrate for the GSSG reductase nor a substrate for GSH transferase. This would explain not only the findings in liver tissue, but also in polymorphonuclear leukocytes which lose glutathione during phagocytosis [20]. In this latter study less than half of the loss of glutathione was recovered in the pool of GSSG and glutathione mixed disulfides.

Metabolic aspects

The amount of protein glutathione mixed disulfides is related to the concentration of GSSG in the cell (Fig. 4). The reaction can be catalysed by thiol transferases, e.g. in the following reaction [2]:

$$\text{prot-SH} + \text{GSSG} \rightleftharpoons \text{prot-SSG} + \text{GSH}$$

Thus, besides being reduced by glutathione reductase and being released into the bile, GSSG appears to be disposed of, to some extent, by way of protein mixed disulfide formation. In the range investigated, a 1:1 relationship between GSSG and glutathione mixed disulfides was observed.

Toxicity of redox-cycling compounds such as paraquat is expressed at several levels. There is not only production of active oxygen species with the often stressed theory of lipid peroxidation as a mechanism of paraquat toxicity, but also severe decrease in the cellular NADPH/$NADP^+$ ratio due to the toxication (redox-cycling) as well as to the detoxication (GSSG reductase) reactions [21] and in the thiol/disulfide ratio due to GSH peroxidase. The increase in mixed disulfides may also entail changes in regulatory functions. Enzymes involved in carbohydrate metabolism are known to be influenced in a manner that leads to stimulation of the pentose phosphate pathway [2].

Acknowledgements—We wish to thank Miss Birgit Gabriel for excellent technical assistance and Mrs. Ursula Rabe for careful preparation of the diagrams. This study was supported by the Deutsche Forschungsgemeinschaft, Schwerpunkt 'Mechanismen toxischer Wirkungen von Fremdstoffen' (Br 778/1-1; Si 255/6-2) and by the Ministerium für Wissenschaft und Forschung, Nordrhein-Westfalen.

REFERENCES

1. N. S. Kosower and E. M. Kosower, *Int. Rev. Cytol.* **54**, 109 (1978).
2. B. Mannervik and K. Axelsson, *Biochem. J.* **190**, 125 (1980).
3. R. Brigelius, R. Lenzen and H. Sies, *Biochem. Pharmac.* **31**, 1637 (1982).
4. P. L. Keeling, L. L. Smith and W. N. Aldridge, *Biochim. biophys. Acta* **716**, 249 (1982).
5. T. P. M. Akerboom and H. Sies, *Methods Enzymol.* **77**, 373 (1982).
6. F. Tietze, *Analyt. Biochem.* **27**, 502 (1969).
7. K. Asaoka and K. Takahashi, *J. Biochem.* **90**, 1237 (1981).
8. T. P. M. Akerboom, M. Bilzer and H. Sies, *J. biol. Chem.* **257**, 4248 (1982).
9. H. Sies, *Methods Enzymol.* **52**, 48 (1978).
10. G. Harisch, J. Eikemeyer and J. Schole, *Experientia* **35**, 719 (1979).
11. D. J. Reed, J. R. Babson, P. W. Beatty, A. E. Brodie, W. W. Ellis and D. W. Potter, *Analyt. Biochem.* **106**, 55 (1980).
12. W. F. Habig, M. J. Pabst and W. B. Jacoby, *J. biol. Chem.* **249**, 7130 (1974).
13. H. Kappus and H. Sies, *Experientia* **37**, 1233 (1981).
14. H. Modig, *Biochem. Pharmac.* **17**, 177 (1968).
15. E. Beutler and C. West, *Analyt. Biochem.* **81**, 458 (1977).
16. P. J. Hissin and R. Hilf, *Analyt. Biochem.* **74**, 214 (1976).
17. G. Harisch and M. F. Mahmoud, *Hoppe-Seyler's Z. physiol. Chem.* **361**, 1859 (1980).
18. J. Isaacs and F. Binkley, *Biochim. biophys. Acta* **497**, 192 (1977).
19. H. Sies, R. Brigelius and T. P. M. Akerboom, in *Functions of Glutathione—Biochemical, Physiological and Toxicological Aspects* (Eds. A. Larsson, S. Orrenius and A. Holmgren), pp. 51–64. Raven Press, New York (1983).
20. B. R. Burchill, J. M. Oliver, C. B. Pearson, E. D. Leinbach and R. D. Berlin, *J. cell. Biol.* **76**, 439 (1978).
21. R. Brigelius. T. P. M. Akerboom and H. Sies, in *Superoxide and Superoxide Dismutase III* (Eds. G. Cohen and R. Greenwald), Vol. II, pp. 51–64. Elsevier North-Holland, Amsterdam (1983).
22. W. Häcki and G. Paumgartner, *Experientia* **29**, 1091 (1973).