Resolution of Glutathione-linked Enzymes in Rat Liver and Evaluation of their Contribution to Disulfide Reduction *via* Thiol—Disulfide Interchange

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Glutathione-linked enzymes in the postmicrosomal fraction of rat liver were resolved and the contribution of these enzymes to disulfide reduction *via* thiol-disulfide interchange was evaluated. The mixed disulfide of cysteine and glutathione (CySSG)*, 5,5′-dithiobis(2-nitrobenzoate) (DTNB), and the mixed disulfide of 3-carboxy-4-nitrobenzenethiol and glutathione (ArSSG) were used as disulfide substrates and glutathione (GSH) was the thiol substrate. Thiol-disulfide interchange involving GSH was normally followed by coupling to glutathione reductase (EC 1.6.4.2), but the reaction between DNTB (or ArSSG) and GSH was measured directly without coupling.

CySSG-thioltransferase activity** (EC 1.8.4.1) was separated into two and DTNB-thioltransferase activity into several components. Glutathione reductase and glutathione *S*-aryltransferase (EC 2.5.1.13) behaved as one and two components, respectively. Aged rat liver preparations contained additional, more acidic species of all these activities except the aryltransferase.

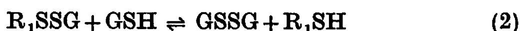
NADPH-linked reduction of DTNB coincided with glutathione reductase activity after all purification steps. No significant contribution to the thiol-disulfide interchange activity in rat liver could be ascribed to glutathione reductase or glutathione *S*-aryltransferase.

The aromatic disulfide substrates, DTNB and ArSSG, are not suitable for the study of CySSG-thioltransferase in crude enzyme-preparations unless the effect of interfering activities can be evaluated.

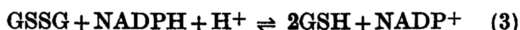
Thiol-disulfide interchange was found to be the major route of DTNB reduction in rat liver. The NADPH-dependent reduction of DTNB described in the literature may be explained by the presence of GSH and glutathione reductase.

In our studies concerning the reduction of low molecular weight disulfides in biological systems the role of glutathione reductase in rat liver cytosol has been evaluated.[1] For disulfides other than glutathione disulfide the main route of enzymatic reduction is composed of thiol-disulfide interchange (eqns. 1, 2) coupled to reduction of GSSG by NADPH and glutathione reductase (eqn. 3).

$$R_1SSR_2 + GSH \rightleftharpoons R_1SSG + R_2SH \quad (1)$$



$$R_1SSG + GSH \rightleftharpoons GSSG + R_1SH \quad (2)$$



$$GSSG + NADPH + H^+ \rightleftharpoons 2GSH + NADP^+ \quad (3)$$

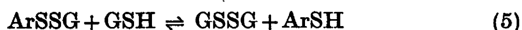
Glutathione (GSH) is the predominant thiol in living cells and probably the main thiol substrate for thioltransferase-catalyzed reactions *in vivo*. Several low molecular weight disulfides have been reported to be reduced enzymatically by GSH *via* a thiol-disulfide interchange, for instance the naturally occurring homocystine,[2] the mixed disulfide of CoA and GSH,[3–5] cystine,[1,3,6–9] the mixed disulfide of cysteine and GSH,[1,3] and others such as thiamine disulfide derivatives [10] and the nonbio-

* Unusual abbreviations: ArSSG, the mixed disulfide of glutathione and 3-carboxy-4-nitrobenzenethiol; CySSG, the mixed disulfide of glutathione and cysteine; $CySSO_3H$, *S*-sulfocysteine; DTNB, 5,5′-dithiobis(2-nitrobenzoate).

** Thioltransferase has recently been introduced as a name for enzymes catalyzing thiol-disulfide interchange.[12,26] These enzymes have earlier been referred to as transhydrogenases.[2]

logical disulfides 5,5′-dithiobis(2-nitrobenzoate) (DTNB) and the mixed disulfide of 3-carboxy-4-nitrobenzene thiol and GSH (ArSSG).[11] The purpose of using DTNB (denoted ArSSAr in eqn. 4 to emphasize its disulfide character) or ArSSG as the disulfide substrate was to obtain a convenient assay method of the thioltransferase activity based on direct spectrophotometric determination of ArSH released as shown in eqns. (4) and (5)

$$\text{ArSSAr} + \text{GSH} \rightleftharpoons \text{ArSSG} + \text{ArSH} \quad (4)$$



$$\text{ArSSG} + \text{GSH} \rightleftharpoons \text{GSSG} + \text{ArSH} \quad (5)$$

To determine whether DTNB could generally replace cystine derivatives in the determination of thioltransferase activity, the activity profiles with these different substrates obtained after column chromatography and isoelectric focusing have been compared.

It has previously been demonstrated that the essence of the enzymatic thiol-disulfide interchange is thiol transfer.[12] Glutathione reductase and glutathione S-aryltransferase also have an element of thiol transfer in their catalytic functions, and the present investigation was undertaken to clarify whether these enzymes contribute to the thioltransferase activity measured in rat liver preparations. The limitations of replacing the glutathione reductase-coupled assay of thioltransferase with direct spectrophotometric measurement using DTNB as substrate as well as the nature of the DTNB-thioltransferase activity are discussed. In addition, DTNB has previously been used to characterize NADPH-dependent reductases with protein disulfides and low molecular weight disulfides as substrates in crude preparations,[9,13–17] but in these studies possible interference by endogenous glutathione reductase, thioltransferase, and GSH, and by the thioredoxin system was not evaluated. It was therefore of interest to see whether NADPH-dependent reduction of DTNB could be explained by a combination of thiol-disulfide interchange according to eqns. (4) and (5) and the reduction of GSSG by NADPH mediated by glutathione reductase.

As regards glutathione S-aryltransferase, it has been suggested that this activity is responsible for the enzymatic reaction of GSH with disulfides,[18] and the relation of this enzyme to CySSG-thioltransferase has therefore also been examined. Preliminary results from this investigation have been previously reported.[19,20]

MATERIALS AND METHODS

The mixed disulfide of cysteine and glutathione was synthesized according to Eriksson and Eriksson,[21] S-sulfocysteine according to Segel and Johnson,[22] and the mixed disulfide of glutathione and 3-carboxy-4-nitrobenzenethiol according to Mannervik.[23] The homogeneity of the compounds in stock solutions was tested by paper electrophoresis [21] before use in the enzymatic experiments, and in no case were any impurities detected.

Glutathione reductase (yeast), NADPH, GSH, and GSSG were obtained from Sigma; 5,5′-dithiobis(2-nitrobenzoic acid) from Aldrich ;3,4-dichloro-1-nitrobenzene from Schuchardt; Ampholine carrier ampholytes from LKB; Sephadex G-25 Fine from Pharmacia Fine Chemicals; and CM-cellulose CM 32 from Whatman.

Separation of rat liver enzymes. Livers from male Sprague-Dawley rats were homogenized in 4 volumes of 0.25 M sucrose and the resulting homogenate was centrifuged at 105 000 g for 60 min. The supernatant was passed through a Sephadex G-25 Fine column which had a volume 5 times the volume of the supernatant. The gel was equilibrated with the eluent required in subsequent experiments. The protein fraction of the effluent was used in the isoelectric focusing and ion-exchange chromatography experiments.

Isoelectric focusing was carried out in a 110 ml column according to the instructions of the manufacturer (LKB). The ampholyte (1 %) covered a pH-range of 3 – 10 (Ampholine No. 8141) in a 0 – 50 % sucrose density gradient. The sample (50 – 100 mg of protein) was desalted on a Sephadex G-25 Fine column (equilibrated with water) and introduced into the middle of the gradient to avoid denaturation by contact with the electrolytes surrounding the electrodes. The isoelectric focusing was run at 4 °C for 36 h at 300 V. The contents of the column were collected in 1.0 – 1.5 ml fractions.

CM-cellulose chromatography was carried out in columns (2 × 10 cm) equilibrated with 10 mM sodium phosphate buffer, pH 6.1 (1 mM EDTA). After introduction of the sample, the column was washed with the phosphate buffer used for equilibration until no protein could be detected in the effluent. The elution was continued with a linear concentration gradient of 0 – 0.2 M NaCl (500 ml total volume) in the original buffer and fractions of 7 – 10 ml were collected.

The protein concentration was calculated on the basis of the absorbance at 260 and 280 nm.[24]

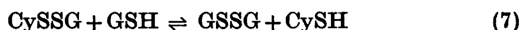
Assays of enzymatic activities. Glutathione reductase was assayed spectrophotometrically

by recording NADPH oxidation at 340 nm. The reaction system contained 1 mM GSSG, 0.1 mM NADPH, 0.17 M sodium phosphate buffer (pH 7.6), 1 mM EDTA, and enzyme (10 – 50 μl) in a total volume of 1 ml. The measurements were made on an LKB 8600 Reaction Rate Analyzer, which maintained the reaction mixture at 30 °C. Reductase activity demonstrable with DTNB as the disulfide substrate was determined in the same reaction system with the substitution of 0.5 mM DTNB for GSSG. The formation of 3-carboxy-4-nitrobenzenethiolate was followed at 412 nm with a recording spectrophotometer.

Glutathione *S*-aryltransferase activity was measured by spectrophotometric determination of *S*-2-chloro-4-nitrophenyl-glutathione using 3,4-dichloro-1-nitrobenzene and GSH as substrates (*cf.* Ref. 25). The determination was made on an LKB 8600 Reaction Rate Analyzer at 340 nm, using an extinction coefficient of 10 mM^{-1} cm^{-1}. The reaction system contained 5 mM GSH, 1 mM 3,4-dichloro-1-nitrobenzene (added as a 20 mM solution in ethanol), 0.17 M sodium carbonate buffer (pH 8.0), and enzyme (50 μl) in a final volume of 1 ml.

Assay of CySSG-thioltransferase was based on the use of GSH and CySSG or GSH and $CySSO_3H$ as substrates and determination of the GSSG formed (as shown in eqns. (6) and (7))

$$CySSO_3^- + GSH \rightleftharpoons CySSG + HSO_3^- \quad (6)$$



$$CySSG + GSH \rightleftharpoons GSSG + CySH \quad (7)$$

by coupling to glutathione reductase (eqn. 3). CySSG and $CySSO_3H$ are interchangeable in this assay system; it has previously been demonstrated by column chromatography and isoelectric focusing experiments that these substrates define the same enzymatic activity (as do, for example, *S*-sulfoglutathione and cystine).[19,20,26] $CySSO_3H$ has the advantage over CySSG of giving a three-fold lower non-enzymatic reaction rate when these substrates are used in the concentrations given below, which result in the same enzymatic reaction rate. The activity was determined spectrophotometrically at 340 nm using a Beckman DB-G spectrophotometer. The reaction system contained 0.5 mM GSH, 0.25 mM CySSG, 0.1 mM NADPH, 0.4 unit of yeast glutathione reductase, 0.125 M sodium phosphate buffer (pH 7.6), 1 mM EDTA, and enzyme (50 – 100 μl) in a final volume of 1 ml. The reaction was started by addition of CySSG (or $CySSO_3H$) 3 min after mixing of the other components.[1] The amount of yeast glutathione reductase used was sufficient to eliminate the influence on the thioltransferase assay of endogenous glutathione reductase in the sample tested. $CySSO_3H$ was used in a concentration of 2.5 mM, when substituted for CySSG. A correction for the spontaneous reaction was made by separate determination of thiol-disulfide interchange in the absence of thioltransferase. Boiled enzyme preparations had no catalytic effect on the reaction.

Measurement of thioltransferase activity using ArSSG or DTNB as the disulfide substrate (ArSSG- or DTNB-thioltransferase activity) was based on the formation of 3-carboxy-4-nitrobenzenethiolate, which was monitored spectrophotometrically at 412 nm (*cf.* Ref. 11). The reaction system contained 0.02 mM GSH, 0.1 mM ArSSG or 0.02 mM DTNB, 0.17 M sodium phosphate buffer (pH 5.5), 1 mM EDTA, and enzyme (50 μl). The reaction velocities were corrected for the contribution of the nonenzymatic reaction.

The formation of 1 μmol/min of product was designated as a unit of enzymatic activity.

RESULTS

Isoelectric focusing of thioltransferase from rat liver. To obtain an overall view of all enzymatic activities of interest in this investigation, a post-microsomal supernatant from homogenates of rat liver was subjected to isoelectric focusing in the pH range of 3 – 10. Fig. 1 shows an experiment with a supernatant dialyzed for 30 h against 1 % glycine (pH 7). Four major peaks of thioltransferase activity were demonstrated using $CySSO_3H$ and GSH as substrates. In other experiments following prolonged dialysis (70 h) only one acidic peak was obtained, apparently at the expense of the two basic components. Only the two basic components were observed when the enzyme sample was chromatographed on Sephadex G-25 before the isoelectric focusing.

Two components of glutathione reductase (not shown) and glutathione *S*-aryltransferase which did not coincide with each other or the other activities tested were detected. DTNB-thioltransferase activity gave four peaks (not shown), two of which partially overlapped the two basic peaks of CySSG-thioltransferase activity.

To test whether the activity obtained with DTNB as the disulfide substrate could be resolved further from the other enzyme activities tested, a more extensive separation of the enzyme activities was undertaken using ion exchange chromatography in combination with isoelectric focusing.

Chromatography of the cytosol fraction of rat liver on CM-cellulose. All enzyme activities studied in the cytosol fraction of fresh rat livers were adsorbed onto CM-cellulose equili-

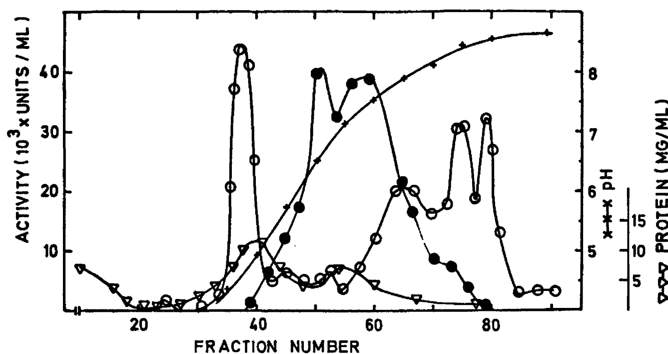


Fig. 1. Isoelectric focusing of the supernatant fraction from rat liver after dialysis for 30 h against 1 % glycine buffer, pH 7. The protein sample, 100 mg, was introduced into the middle of the 0 – 50 % sucrose gradient containing 1 % Ampholine (No. 8141), pH 3 – 10. Isoelectric focusing was carried out at 4 °C for 36 h at 300 V. The contents of the column were collected in 1.0 – 1.5 ml fractions. ○, CySSG-thioltransferase; ●, GSH *S*-aryltransferase × 1:20.

brated with 10 mM sodium phosphate buffer, pH 6.1, containing 1 mM EDTA. Elution with a salt gradient resulted in two peaks of CySSG-thioltransferase activity (Fig. 2), the relative sizes of which were dependent on the experimental conditions. This variable elution pattern indicated that the two activities might be interconvertible forms of a single enzyme. This possibility was the subject of a separate investigation in which evidence for a GSH-dependent interconversion of the two forms was obtained [27] (see also Ref. 26). A similar variable elution pattern was not seen for any of the other activities studied. Aged preparations contained additional activities which were not retained by the column and which presumably were identical with the acidic degradation products observed in the isoelectric focusing experiments.

Glutathione reductase appeared as a single component which had its peak in a fraction containing the maximal activities of thioltransferase, measured with ArSSG and DTNB as disulfide substrates. The activities obtained with ArSSG and DTNB were usually distributed in three distinct peaks which essentially coincided for the two substrates. The major peaks of the two activities had shoulders which overlapped with fractions containing the maxima of the CySSG-thioltransferase activity. The glutathione *S*-aryltransferase activity had two peaks which partially coincided with the thioltransferase activity.

Isoelectric focusing of fractions obtained from CM-cellulose chromatography. Fractions 37 and 39 from the CM-cellulose chromatography depicted in Fig. 2 were subjected to isoelectric focusing in an attempt to resolve the different enzymatic activities. The resolution of fraction No. 37 is shown in Fig. 3. Glutathione reductase, glutathione *S*-aryltransferase, and CySSG-thioltransferase activities were clearly resolved. DTNB-thioltransferase activity is separated into three peaks coinciding with the peaks of glutathione reductase, glutathione *S*-aryltransferase, and CySSG-thioltransferase.

Isoelectric focusing of fraction No. 39, containing low CySSG-thioltransferase activity, is illustrated in Fig. 4. CySSG-thioltransferase activity is less stable than the other activities (see Refs. 6, 15, and 28) and could not be detected after the isoelectric focusing. Glutathione reductase appeared as a single component separated from the other activities. The glutathione *S*-aryltransferase activity appeared as one minor and two major components. The enzymatic activity demonstrated with DTNB as disulfide substrate was separated into one major and two minor peaks. The coincidence of glutathione *S*-aryltransferase and DTNB-thioltransferase activities (Figs. 3 and 4) will be considered further in the Discussion.

In a different series of experiments the fraction from the CM-cellulose column containing the second component of CySSG-thioltransferase (corresponding to fraction No. 41 in Fig. 2) was analyzed by isoelectric focusing (Fig. 5).

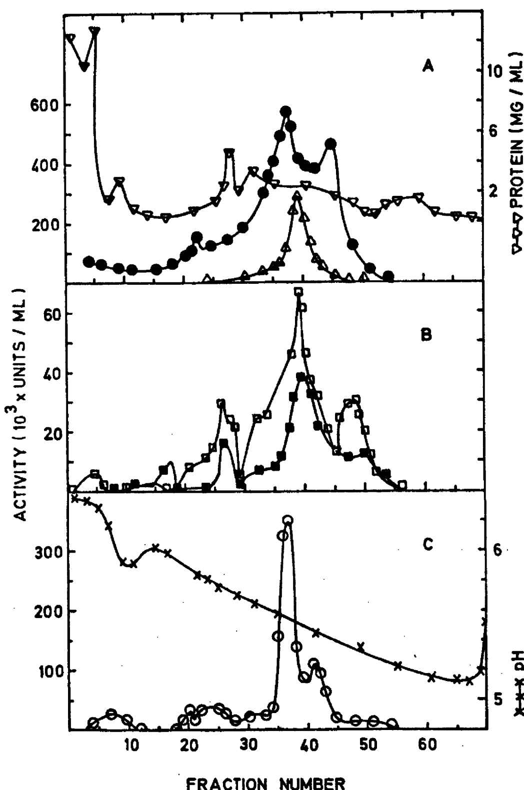


Fig. 2. Fractionation of the supernatant fraction from rat liver by CM-cellulose chromatography. To obtain the protein sample in the same buffer conditions prevailing in the equilibrated CM-cellulose, the supernatant was passed through five volumes of Sephadex G-25 equilibrated with 10 mM sodium phosphate buffer (pH 6.1), 1 mM EDTA. The protein effluent was applied to the CM-cellulose column (2 × 10 cm). The column was washed with the buffer used for equilibration till no protein was eluted. The elution was continued with a linear concentration gradient of 0 – 0.2 M NaCl (500 ml total volume) in the original buffer and fractions of 7 – 10 ml were collected. The enzymatic activities are: (A) ●, GSH *S*-aryltransferase; △, glutathione reductase (activity × 1:5); (B) □, DTNB-thioltransferase activity; ■, ArSSG-thioltransferase activity; (C) ○, CySSG-thioltransferase.

The results were essentially the same as those depicted in Fig. 3, but some of the CySSG-thioltransferase activity was found in the low pH-range of the gradient, indicating degradation due to prolonged dialysis before isoelectric focusing. Furthermore, the reductase activity obtained with DTNB and NADPH (in the absence of added thiols), which was not separated from glutathione reductase by CM-cellulose chromatography, coincided exactly with the glutathione reductase activity.

In addition, we investigated the possibility that thioltransferase activities with DTNB or ArSSG were catalyzed by the same proteins catalyzing the other activities tested. Isoelectric focusing of a fraction corresponding to No. 26 in Fig. 2, the first of the three major peaks containing DTNB-thioltransferase activity, showed high isoelectric points for the components of DTNB-thioltransferase, which were resolved from the other activities tested (Fig. 6). Furthermore, low activity was obtained when ArSSG was used as the substrate in assaying fractions containing high glutathione *S*-aryltransferase and CySSG-thioltransferase activities.

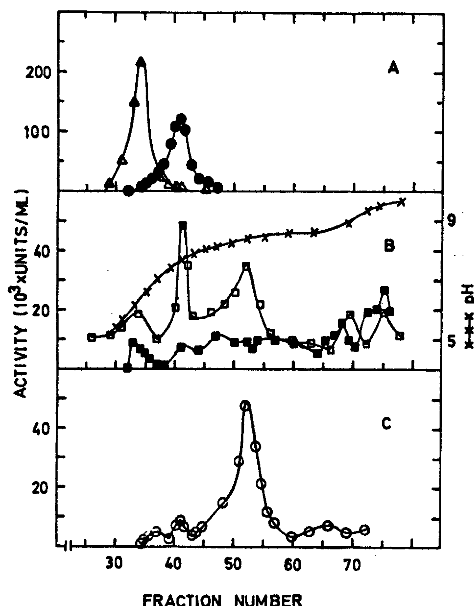


Fig. 3. Isoelectric focusing of fraction No. 37 from the CM-cellulose chromatography depicted in Fig. 2. The symbols for enzymatic activities are explained in the legend to Fig. 2. Further information is supplied in Fig. 1 and Materials and Methods.

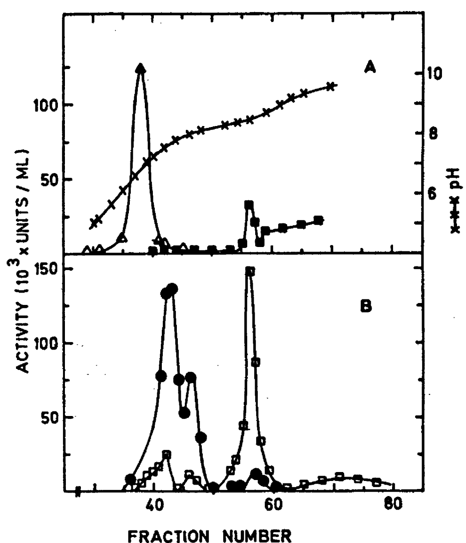


Fig. 4. Isoelectric focusing of fraction No. 39 from the CM-cellulose chromatography depicted in Fig. 2. The symbols are given in Fig. 2. Glutathione reductase (activity x 1:10). Further information is supplied in Fig. 1 and Materials and Methods.

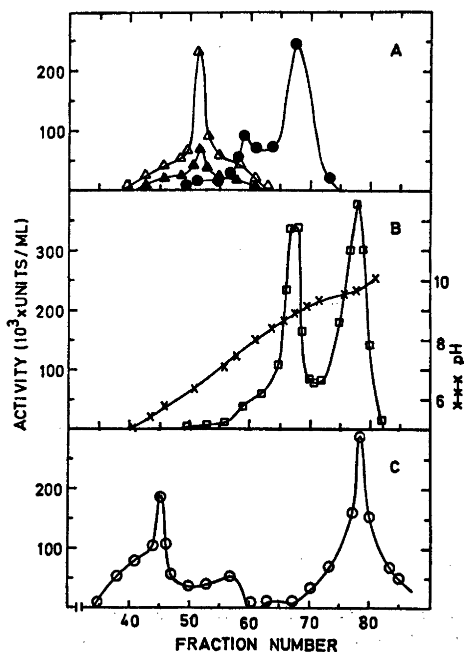


Fig. 5. Isoelectric focusing of a fraction from CM-cellulose chromatography corresponding to fraction No. 41 in Fig. 2. ▲, NADPH-DTNB oxidoreductase; △, glutathione reductase (activity x 1:40), ●, GSH-*S*-aryltransferase (activity x 1:4). The other symbols are given in Fig. 2. Further information is supplied in the legend to Fig. 1 and Materials and Methods.

DISCUSSION

The results of the present investigation demonstrate that the cytosol fraction of rat liver contains at least two forms of thioltransferase active with CySSG (or $CySSO_3H$). These components are distinguishable by chromatography on CM-cellulose columns or by isoelectric focusing. The two activities, after being separated by ion exchange chromatography, are essentially homogeneous in subsequent isoelectric focusing experiments (*cf.* Figs. 3 and 5). Upon aging of the supernatant, additional, more acidic forms appear. (For instance, the experiment demonstrated in Fig. 5 shows activity below pH 8 in the gradient presumably due to degradation of the sample during dialysis before the isoelectric focusing.) It could be established (*cf.* Figs. 3 and 5) that no significant contribution to the CySSG-thioltransferase activity could be ascribed either to glutathione reductase (*cf.*

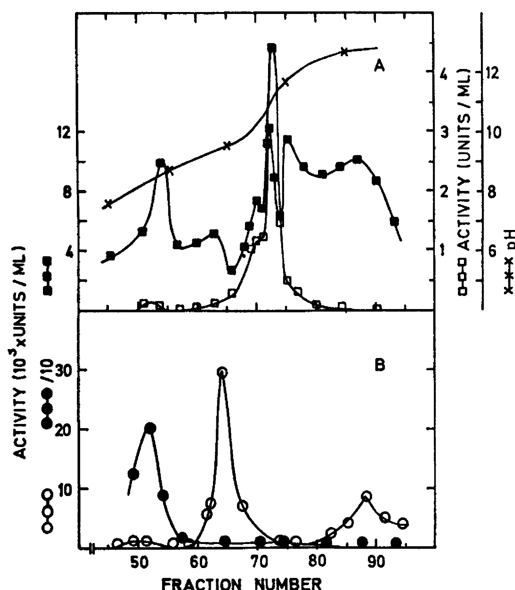


Fig. 6. Isoelectric focusing of a fraction from CM-cellulose chromatography corresponding to the first peak in Fig. 2 B containing fractions Nos. 20 – 29. The substrate concentrations used for measuring DTNB- and ArSSG-thioltransferase activities are: GSH, 50 μM; DTNB, 50 μM; and ArSSG, 135 μM. GSH *S*-aryltransferase (activity x 1:10). The symbols used are explained in the legend to Fig. 2. Further information is supplied in the legend to Fig. 1 and Materials and Methods.

Ref. 11) or to glutathione *S*-aryltransferase, which has been stated to be identical with an enzyme catalyzing the thiol-disulfide interchange involving GSH and thiamine disulfide.[18]

The fact that several different enzymes can use DTNB and GSH as substrates, in combination with the limited resolution of the enzyme activities, explains the unusual shape of the peaks of the DTNB activity profile depicted in Fig. 2.

The third peak of DTNB-dependent activity in Fig. 2 B was not characterized further, but is probably a separate activity, since the activities of all other enzymes examined were low in these fractions from the column. Thus, all three peaks of DTNB activity seemed to contain a component which was distinct from glutathione reductase, glutathione *S*-aryltransferase, and CySSG-thioltransferase. Three major peaks of DTNB-dependent activity have been seen in all experiments, and the first has been found to increase upon aging of the rat liver preparation.

It is obvious from Figs. 2 and 6 that the glutathione reductase-coupled system for determination of thioltransferase activity catalyzing thiol-disulfide interchange of cystine and glutathione derivatives cannot without restrictions be replaced by the assay method based on the thiolysis of DTNB [11] when a crude enzyme preparation is used. In conclusion, a number of protein components in the supernatant fraction from rat liver catalyze the reduction of non-biological aromatic disulfides by GSH.

Glutathione reductase normally appeared as a single component in chromatographic experiments. The reductase activity obtained with DTNB and NADPH as substrates coincided with the glutathione reductase activity in all experiments (*cf.* Fig. 5). This contrasts with the finding of Tietze that the DTNB- and GSSG-reducing activities can be separated by chromatography on DEAE-cellulose.[15]

The glutathione *S*-aryltransferase activity appeared as two components which are separable from the major components of the other activities tested (*cf.* Figs. 1, 2, 4 and Ref. 29). However, isoelectric focusing did not resolve overlapping peaks of glutathione *S*-aryltransferase and DTNB-thioltransferase activity (*cf.* Figs. 3, 4, and 5) or glutathione *S*-aryltransferase and ArSSG-thioltransferase activity (Fig. 6). This finding indicates that glutathione *S*-aryltransferase, which is known to have a broad substrate specificity,[25] can utilize DTNB and ArSSG as substrates. The nature of the reactions with these substrates has not been studied, and although the original assumption was that a thiol-disulfide interchange was measured, it cannot be excluded that arylation of GSH occurred. It is known that DTNB can arylate sulfhydryl groups nonenzymatically.[30]

The results in Fig. 4 also seem to indicate that the component showing the highest thioltransferase activity with DTNB and GSH as substrates is able to catalyze the arylation of GSH with 3,4-dichloro-1-nitrobenzene.

A general conclusion based on the present investigation is that assay of either glutathione reductase, glutathione *S*-aryltransferase, or CySSG-thioltransferase is not interfered with by

the other two enzymes. The glutathione *S*-aryltransferase (*cf.* Refs. 18 and 29) and the CySSG-thioltransferase activities are present in at least two separable forms. The different forms of both of these two enzyme activities seem to be capable of catalyzing the reaction of DTNB and ArSSG with GSH. DTNB-thioltransferase activity is also associated with several protein components distinct from the other enzyme activities measured (Figs. 2, 4, and 6). Before concluding definitely that these activities are thioltransferases, it is desirable to further characterize them, bearing in mind that DTNB is a compound not normally found *in vivo*.

The two components of the CySSG-thioltransferase activity are interconvertible, as is demonstrated in a following paper.[27] This finding indicates that these are different forms of a single enzyme. This activity is responsible for the enzymatic reduction in rat liver of a variety of low molecular weight disulfides (see Introduction), as well as for the reduction of *S*-sulfo-derivatives of, for example, cysteine or GSH.[26] CySSG-thioltransferase is present in the cytosol [1] and is clearly distinguishable from the enzyme ("GSH-insulin transhydrogenase") catalyzing thiol-disulfide interchange between GSH and insulin or other polypeptide disulfides.[31,32] The latter enzyme is also present in rat liver [33,34] and is probably localized in the microsomal fraction.[15,35,36] The conclusion that the two enzymes are different is further supported by the lack of activity of purified CySSG-thioltransferase with 0.25 mM GSH and 0.25 mM insulin or oxytocin in the glutathione reductase-coupled system (S. Eriksson, unpublished experiments). On the other hand, the CySSG-thioltransferase is probably identical with the thiol-disulfide "transhydrogenase" of rat tissues active with low molecular weight substrates.[3,7,15] This latter enzyme was originally described by Racker under the name of glutathione-homocystine transhydrogenase [2] and has subsequently also been purified from yeast.[6]

The enzyme-catalyzed reaction between GSH and DTNB has, with the exception of the activity observed with partially purified CySSG-thioltransferase,[11] not previously been described. It seems possible that the enzymatic activity observed with GSH and thiamine disulfides as substrates may be of a similar nature.[10,18] A partially purified preparation of the latter activity was not active with cystine, cystamine, or homocystine as substrates, which was taken as evidence that this activity is not identical with CySSG-thioltransferase.[10] The thioltransferase activities obtained with DTNB and that described for thiamine disulfides are similar in that some of the components separated by ion-exchange chromatography or isoelectric focusing coincide with peaks of glutathione *S*-aryltransferase activity (Figs. 3, 4, and 5; *cf.* Ref. 18).

It is clear that the high redox potential of DTNB makes it more reactive than alkyl disulfides. Therefore, it is not unexpected that several components of rat liver are able to catalyze the reaction between thiols and DTNB (*cf.* Figs. 3 and 5). Whether the activity demonstrated with DTNB has any physiological significance remains to be seen.

Pyridine nucleotide-dependent reduction of DTNB has previously been observed in several enzymatic systems. In bacteria and bacterial spores a reductase has been identified which is more active with DTNB than with any other disulfide (including GSSG) tested.[13,14] Multicomponent systems,[37,38] probably identical with the thioredoxin system,[39] capable of NADPH-dependent reduction of disulfides are also active with DTNB. A similar reduction of DTNB which may also be partially linked to thioredoxin has been detected in rat liver.[15–17] Furthermore, it is known that glutathione reductase can directly catalyze the reduction of DTNB, although this activity is relatively low.[40,41] In the present investigation the only NADPH-dependent activity detected after isoelectric focusing (Fig. 5) or CM-cellulose chromatography seems to be the latter activity; the thioredoxin system is probably not functional due to separation of thioredoxin and thioredoxin reductase. Finally, it should be observed that the discussion in the literature of the NADPH-dependent reduction of DTNB [9,15–17] has ignored the reactions between DTNB and GSH (eqns. 4, 5), which can be coupled to glutathione reductase (eqn. 3). The present investigation shows that this thiol-disulfide interchange is enzymatic. Failure to properly evaluate the contribution of corresponding thiol-disulfide interchange reactions has previously limited the understanding of the nature of "cystine reductase" [42–44] (*cf.*

Ref. 45), "*S*-sulfoglutathione reductase",[46,47] (*cf.* Ref. 48) and "coenzyme A-glutathione mixed disulfide reductase"[49–52] (*cf.* Refs. 5 and 26).

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