COMPETITION BETWEEN TRANSPORT OF GLUTATHIONE DISULFIDE (GSSG) AND GLUTATHIONE *S*-CONJUGATES FROM PERFUSED RAT LIVER INTO BILE

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1. Introduction

Glutathione is transported out of the liver in different forms and into different compartments (overview [1]). While the reduced form, GSH, is mainly released across the sinusoidal membrane into plasma, the oxidized form, GSSG, is preferentially released across the canalicular membrane into the biliary space [2]. Also, the glutathione *S*-conjugates, thioethers known as precursor of the mercapturic acids, are preferentially released into the biliary space [3,4].

Properties of these transport systems are known only to a limited extent [5,6], and it has been suggested that the transport of glutathione disulfide and of glutathione *S*-conjugates may have common features, viewing the disulfide as a glutathione homoconjugate [1]. Here, we investigate whether transport of GSSG is related to the transport of *S*-conjugates, and vice versa. Stimulation of biliary GSSG release was achieved by the addition of hydroperoxide or of nitrofurantoin, conditions exhibiting a close correlation between the intracellular GSSG content and the rate of biliary GSSG release [6]. Stimulation of biliary *S*-conjugate release was effected by the addition of substrates for glutathione *S*-transferase activities, e.g., 1-chloro-2,4-dinitrobenzene or diethyl maleate [3,4].

2. Materials and methods

2.1. *Liver perfusion, sampling and processing*

Non-recirculating hemoglobin-free perfusion of livers from male Wistar rats (180–220 g body wt) fed on stock diet (Altromin, Lage) was performed as in [7] except that the liver was kept in situ throughout the experiment. The bile duct was cannulated using a polyethylene tube of 0.4 mm inner diameter and 50 mm length [6].

After 30 min (substrate-free) perfusion, additions were made into the entering perfusate directly before the portal vein, using infusion pumps. For measurement of intracellular glutathione, experiments were terminated at 43 min perfusion by freeze-clamping the liver tissue. The frozen liver tissue was processed for the determination of GSSG as detailed [8]. Bile samples were collected at 3 or 5 min intervals directly into Eppendorf cups containing 30 μl 5% metaphosphoric acid [6,9].

2.2. *Assays*

GSSG was assayed by following the oxidation of NADPH at 340–400 nm in a Sigma ZWS 11 dual-wavelength spectrophotometer after addition of GSSG reductase (cf. [8]). Values for intracellular GSSG were corrected for extracellular (canalicular) GSSG, assuming a bile volume of 2.3 μl/g liver wet wt [10]. *t*-Butyl hydroperoxide was determined in a coupled assay using GSH peroxidase and GSSG reductase [11]. *S*-2,4-dinitrophenyl-glutathione was measured by absorbance at 334 nm in an Eppendorf photometer using $\epsilon = 9.6\ mM^{-1} \cdot cm^{-1}$ [4,12].

2.3. *Materials*

Bovine erythrocyte GSH peroxidase was a gift from Dr A. Wendel (Tübingen) and *t*-butyl hydroperoxide was a gift from Peroxidchemie München (Höllriegelskreuth). All other enzymes and coenzymes were obtained from Boehringer (Mannheim); DTNB and NEM were purchased from Merck (Darmstadt), and 1-chloro-2,4-dinitrobenzene, diethylmaleate and nitrofurantoin from Sigma (München).

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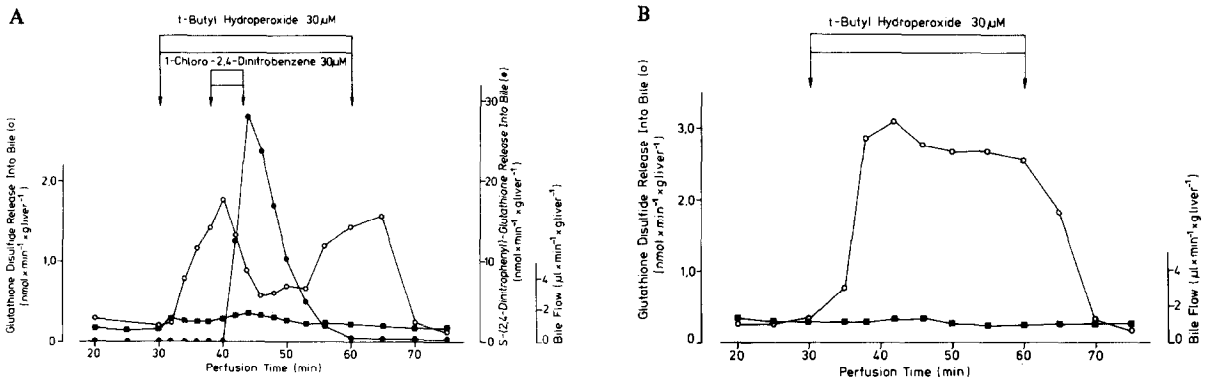

Fig.1. Bile flow (▪) and biliary release of glutathione disulfide (○) and *S*-(2,4-dinitrophenyl)-glutathione (•) in perfused rat liver. GSSG release was stimulated by infusion of *t*-butyl hydroperoxide and release of the glutathione conjugate was effected by infusion of 1-chloro-2,4-dinitrobenzene for 5 min during the time indicated (A). In the control experiment (B) the latter was omitted.

3. Results

3.1. *Restriction of GSSG transport into bile during biliary* S-*conjugate transport*

Addition of a substrate for GSH peroxidase, *t*-butyl hydroperoxide, leads to an increased biliary release of GSSG due to an enhanced intracellular production of GSSG [2,6]. Transport of GSSG into bile is increased several-fold in the presence of *t*-butyl hydroperoxide at 30 µM over 30–60 min perfusion (fig.1). In fig.1A, a substrate for glutathione *S*-transferase, 1-chloro-2,4-dinitrobenzene, was added at 30 µM over 5 min, leading to a substantial decrease in biliary GSSG output concomitant with the release of the corresponding conjugate, *S*-(2-4-dinitrophenyl)-glutathione. Bile flow was slightly increased during the presence of 1-chloro-2,4-dinitrobenzene.

Thus, there is competition between biliary output of GSSG and *S*-conjugate. That this is due to a competition at the transport step across the canalicular membrane is supported by the following:

(i) The omission of the interval with 1-chloro-2,4-dinitrobenzene shows that stable conditions of GSSG transport are maintained throughout the period of perfusion with *t*-butyl hydroperoxide (fig.1B).

(ii) The rate of GSSG release returns to the initial level after withdrawal of 1-chloro-2,4-dinitrobenzene, indicating that sufficiently high intracellular GSH is present throughout the experimental period and that general damage to the transport system does not occur.

(iii) The effect is observed also when GSSG release is stimulated by addition of nitrofurantoin (fig.2) an inhibitor of glutathione reductase, which leads to an increase in intracellular GSSG concomitant with the increased rate of biliary GSSG release [6].

3.2. *Intracellular GSSG during biliary* S-*conjugate transport in the presence of* t-*butyl hydroperoxide or nitrofurantoin*

The above effects are not due to reaction steps proximal to transport across the canalicular membrane. Decrease in GSSG release by the addition of 1-chloro-2,4-dinitrobenzene is not simply caused by an effect on the rate of intracellular GSSG generation (fig.3). When the intracellular GSSG contents are plotted against biliary GSSG release, there is a significant decrease in the slope in the presence of 1-chloro-2,4-dinitrobenzene. This is observed both with *t*-butyl hydroperoxide as a GSSG generator (fig.3A), with

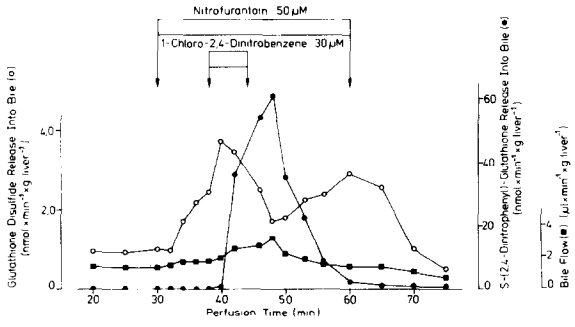

Fig.2. Bile flow (▪) and biliary release of glutathione disulfide (○) and *S*-(2,4-dinitrophenyl)-glutathione (•) upon infusion of nitrofurantoin and 1-chloro-2,4-dinitrobenzene.

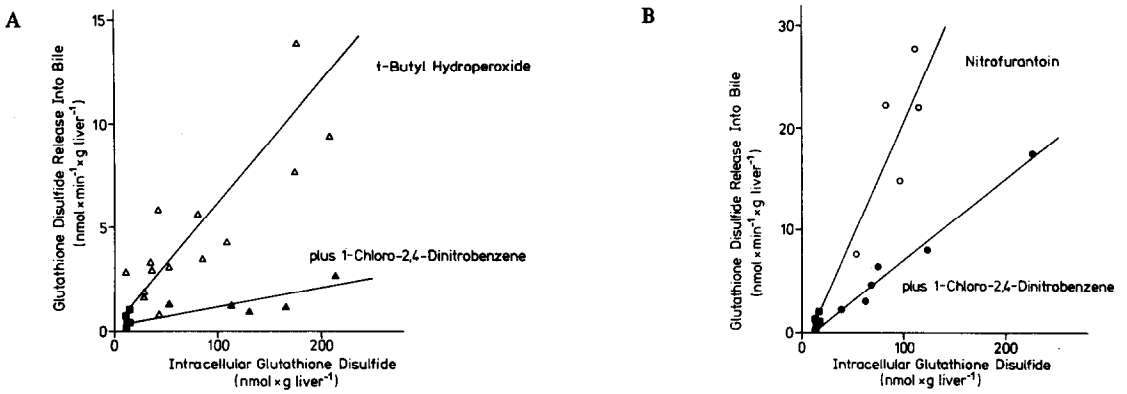

Fig.3. Relationship between the rate of GSSG release into bile and the intracellular GSSG content in the absence and presence of 1-chloro-2,4-dinitrobenzene (30 μM; added at 38 min). GSSG release was stimulated by addition of *t*-butyl hydroperoxide (A) or nitrofurantoin (B) at 30 min. Freeze stop was at 43 min. For other experimental details see section 2. Each point represents 1 perfusion expt: controls (■); *t*-butyl hydroperoxide (30–100 μM) (△); *t*-butyl hydroperoxide (30–100 μM) + 1-chloro-2,4-dinitrobenzene (30 μM (▲); nitrofurantoin (50–150 μM) (○); nitrofurantoin (50–150 μM) + 1-chloro-2,4-dinitrobenzene (30 μM) (●).

nitrofurantoin (fig.3B), and with benzylamine (1 mM), a substrate for monoamine oxidase (not shown).

3.3. *Restriction of* S*-conjugate transport into bile during stimulated GSSG transport*

The converse experiment to that of fig.1A demonstrates a mutual restriction of *S*-conjugate transport and GSSG transport (fig.4). The concentrations used in fig.4 were low for 1-chloro-2,4-dinitrobenzene and high for *t*-butyl hydroperoxide. In a similar experiment with 30 μM hydroperoxide only little effect on *S*-conjugate release was observed (not shown), indicating that apparently the *S*-conjugate is transported more effectively and probably has a lower K_i for GSSG transport than the K_i of GSSG for *S*-conjugate transport.

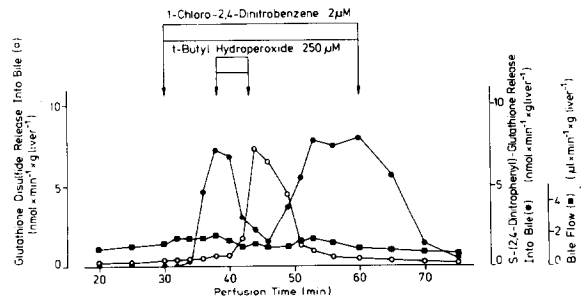

Fig.4. Effect of *t*-butyl hydroperoxide infusion on biliary release of *S*-(2,4-dinitrophenyl)-glutathione: bile flow (■); GSSG release (○); *S*-(2,4-dinitrophenyl)-glutathione release (●).

Throughout the experimental period in fig.4 there is a substantial excess of glutathione *S*-transferase activity, since the total cellular capacity amounts to 120 μmol *S*-conjugate formed . min^{-1} . g $liver^{-1}$ [4], and at a rate of *t*-butyl hydroperoxide infusion of 1 μmol . min^{-1} . g^{-1} there is complete reduction to the alcohol, indicated by practically zero hydroperoxide concentrations in the effluent perfusate [11].

In further experiments, *S*-(2,4-dinitrophenyl)-glutathione transport into bile was also diminished when another substrate for *S*-transferases, diethyl maleate (30 μM), was infused (not shown). This indicates that also between *S*-conjugates there is mutual competition of biliary *S*-conjugate release; however, since competition at the transferase might complicate the interpretation of such effects, this was not further investigated here.

4. Discussion

This work demonstrates mutual competition between transport of GSSG and transport of glutathione *S*-conjugates from perfused rat liver into bile. During the transport of *S*-conjugate there is an increase in intracellular GSSG (fig.3), explainable as a back-up consequential to inhibition at the transport step. Such observations were not obtained in a study with isolated hepatocytes [5]. Since the morphology of the biliary compartment is lost with isolated cells,

the intact liver is probably better suited for studies of biliary transport.

With the general mechanism of bile formation being far from understood (overview [13]) mechanistic conclusions regarding biliary transport of the glutathione derivatives would be premature. However, the mutual interference of the disulfide and the *S*-conjugate transport indicate a close relationship between them, be it either upon entry into biliary vesicles in the cytosol near the canalicular membrane or else at the actual transport step across the canalicular membrane. Thus, the question of whether there exist one or several carrier systems for these different compounds must be left open.

The high calculated biliary/cytosolic concentration gradient for GSSG [6] is consistent with an active transport system of GSSG, as described in erythrocytes [14] and erythrocyte vesicles [15]. An energy-dependent transport of *S*-(2,4-dinitrophenyl)-glutathione was also reported for intact erythrocytes [16] but this was not seen in [17]. Competition between *S*-conjugate and GSSG has not been studied yet in the erythrocyte system.

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References

[1] Sies, H., Wahlländer, A., Waydhas, C., Soboll, S. and Häberle, D. (1980) Adv. Enz. Regul. 18, 303–320.
[2] Sies, H., Wahlländer, A. and Waydhas, C. (1978) in: Functions of Glutathione in Liver and Kidney (Sies, H. and Wendel, A. eds) pp. 120–126, Springer, Berlin, New York.
[3] Barnhart, J. L. and Combes, B. (1978) J. Pharmacol. Exp. Ther. 206, 614–623.
[4] Wahlländer, A. and Sies, H. (1979) Eur. J. Biochem. 96, 441–446.
[5] Eklöw, L., Thor, H. and Orrenius, S. (1981) FEBS Lett. 127, 125–128.
[6] Akerboom, T. P. M., Bilzer, M. and Sies, H. (1982) J. Biol. Chem. 257, in press.
[7] Sies, H. (1978) Methods Enzymol. 52, 48–59.
[8] Akerboom, T. P. M. and Sies, H. (1981) Methods Enzymol. 77, 373–382.
[9] Eberle, D., Clarke, R. and Kaplowitz, N. (1981) J. Biol. Chem. 256, 2115–2117.
[10] Häcki, W. and Paumgartner, G. (1973) Experientia 29, 1091–1093.
[11] Sies, H. and Summer, K. H. (1975) Eur. J. Biochem. 57, 503–512.
[12] Habig, W. F., Pabst, M. J. and Jakoby, W. B. (1974) J. Biol. Chem. 249, 7130–7139.
[13] Boyer, J. L. (1980) Physiol. Rev. 60, 303–326.
[14] Srivastava, S. K. and Beutler, E. (1974) J. Biol. Chem. 244, 9–16.
[15] Kondo, T., Dale, G. L. and Beutler, E. (1980) Proc. Natl. Acad. Sci. USA 77, 6359–6362.
[16] Board, P. G. (1981) FEBS Lett. 124, 163–165.
[17] Awasthi, Y. C., Garg, H. S., Dao, D. D., Partridge, C. A. and Srivastava, S. K. (1981) Blood 58, 733–738.