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DIURNAL FLUCTUATION AND PHARMACOLOGICAL ALTERATION OF MOUSE ORGAN GLUTATHIONE CONTENT

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Abstract—Mouse liver glutathione content showed a diurnal variation with a maximum GSH + 2 GSSG content at 6 to 10 a.m. of 62 ± 8 nmole per mg protein and a minimum of 42 ± 7 at 6 p.m. Starvation for more than 24 hr decreased the hepatic glutathione content to 22 ± 3 nmole/mg protein and abolished the diurnal rhythm. Artificial reversal of the feeding habit of the animals reversed the diurnal rhythm. Kidney, spleen and lung glutathione contents showed no such rhythm. The organ glutathione content decreased by 50% or more upon starvation. The increase of the liver glutathione content by injection of either free or liposomally entrapped GSH to starved animals was not dependent on the time of administration. The physiological maximum level could not be exceeded by this treatment. It was not possible to influence the glutathione content of kidney, lung or intestine by glutathione injections in either form. Intravenous injections of equimolar doses of 2,3-dimercaptopropanol, 2-mercaptoethanesulfonic acid, *N*-2-mercaptopropionylglycine, D-penicillamine, or cysteamine did not lead to any significant change in liver, kidney, spleen or lung glutathione contents 2 hr after administration. Intravenously given *N*-acetylcysteine, methionine, GSH or GSSG restored liver glutathione levels of starved animals to the contents observed in the fed state. The diurnal hepatic variation of GSH caused by the food intake habit of the animals may limit the capacity of the intracellular detoxication system.

The tripeptide glutathione (GSH) is one of the major detoxication factors of the liver. It serves as a substrate for the GSH-peroxidase redox system in order to remove peroxides as well as for glutathione transferases in order to form pre-mercapturic acids as primary phase-II-adducts of many different xenobiotics. The detoxication potential of the liver is intimately connected to the glutathione status of the organ [1].

Several investigators have noted that the hepatic glutathione content undergoes marked diurnal fluctuations and possibly depends on the food intake of animals [2–4]. It has already been shown at a hepatic GSH content near the diurnal minimum, that rats were more sensitive to the lethal and hepatoxic effects of 1,1-dichloroethylene [5] and paracetamol [4].

Many attempts have been undertaken to enhance the hepatic glutathione content with the goal to fortify the defence capacity of the organ. Since extracellular GSH is impermeable to the liver [6] and the latter has a very short half-life [7] due to its rapid renal degradation, administration of the compound itself proved to be unsuitable for a stable increase in liver glutathione [8]. A more promising approach was to supply the body with liver glutathione precursors, e.g. methionine [8], *N*-acetylcysteine [9] and other sulfhydryl-containing pharmacologically active compounds. Recently, we reported that intravenous liposomally entrapped glutathione leads not only to an enhancement of the liver GSH content within 2 hr but also results in an optimal protection of the organ in mice against severe hepatic injury evoked by high doses of paracetamol [10]. We also reported in an earlier study that mice are much more susceptible to the adverse effect of this drug when they are at the minimum of their diurnal GSH content variation [11]. This situation can be brought about by starvation or by feeding a protein-free diet for 2 days [12]. The interrelationship between the natural diurnal liver GSH fluctuation, its dependence on the food intake rhythm, or its ability to incorporate different pharmaceutical forms of GSH or GSH precursors has not been studied in a given species from a common standpoint. Therefore we addressed this problem in different mouse organs from which many biochemical and pharmacological data are already available. We report here that a diurnal rhythm in GSH content in the liver is characteristically dependent on the feeding rhythm, while that in the kidney and lungs is sensitive to starvation. Evidence for the pharmacological manipulation of liver GSH is presented.

MATERIALS AND METHODS

Chemical. 2,3-Dimercaptopropanol (BAL), 2-mercaptoethanesulfonic-acid (Mesna), *N*-2-mercaptopropionylglycine (Thiola), reduced and oxidized glutathione were purchased from Sigma Chemie GmbH (München, F.R.G.). D-Penicillamine and cysteamine were obtained from Fluka AG (Buchs, Switzerland), L-methionine from Serva AG (Heidelberg, F.R.G.), *N*-acetyl-L-cysteine and glutathione

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reductase from Boehringer GmbH (Mannheim, F.R.G.) and all other substances were from Merck AG (Darmstadt, F.R.G.). Liposomal GSH was prepared as described [10].

Animal treatment. Male albino mice weighing 25–30 g were used in all experiments. The animals were kept on a standard laboratory pelleted diet (Altromin, Lage, F.R.G.) in an environmentally controlled room. A group of 50 mice had free access to food and water only from 7 a.m. to 5 p.m. and were fasted overnight. This feeding procedure was maintained for 3 weeks.

At specified times of the day, groups of 6 animals were killed and the organs were perfused with ice cold saline via the left ventricle for 20 sec. The organs were immediately frozen in liquid nitrogen and stored at −30°. After homogenization in 3% metaphosphoric acid glutathione content was determined by the method of Tietze [13].

All animals treated with sulfhydryl compounds were fed a 10% (w/v) liquid sucrose diet for 48 hr prior to the experiment. At 5–7 p.m. solutions of the compounds in saline were injected into the tail vein of the animals at doses of 0.5 mmol/kg body weight. The animals were killed 2 hr later.

Statistics. Results are expressed as mean values ± standard deviation (S.D.). The data were analysed by Student's *t*-test. $P \leq 0.05$ was considered to be significant.

RESULTS

Data in Fig. 1 show the time course of the glutathione content of mouse organs over a 24-hr period under different nutritional conditions. Only in the liver was a significant diurnal variation of glutathione in fed animals observed. Starvation had a pronounced effect on the glutathione content also in spleen, kidney and lung. An apparently minimal content was reached in the liver after 24 hr starvation while the other organs showed a further decrease in the tripeptide after 48 hr starvation. In all organs investigated no further changes in glutathione content were observed after 72 hr (data not shown in the figure).

The food intake of night-active rodents is very low during the light period. Thus it seemed likely that the hepatic diurnal rhythm might be a consequence of the organ's supply with glutathione precursor amino acids. Also, we had observed that feeding a pure sucrose or palmitin diet led to the same decreased glutathione levels in liver as those following starvation. Therefore we artificially reversed the food intake habit of the animals. Figure 2 illus-

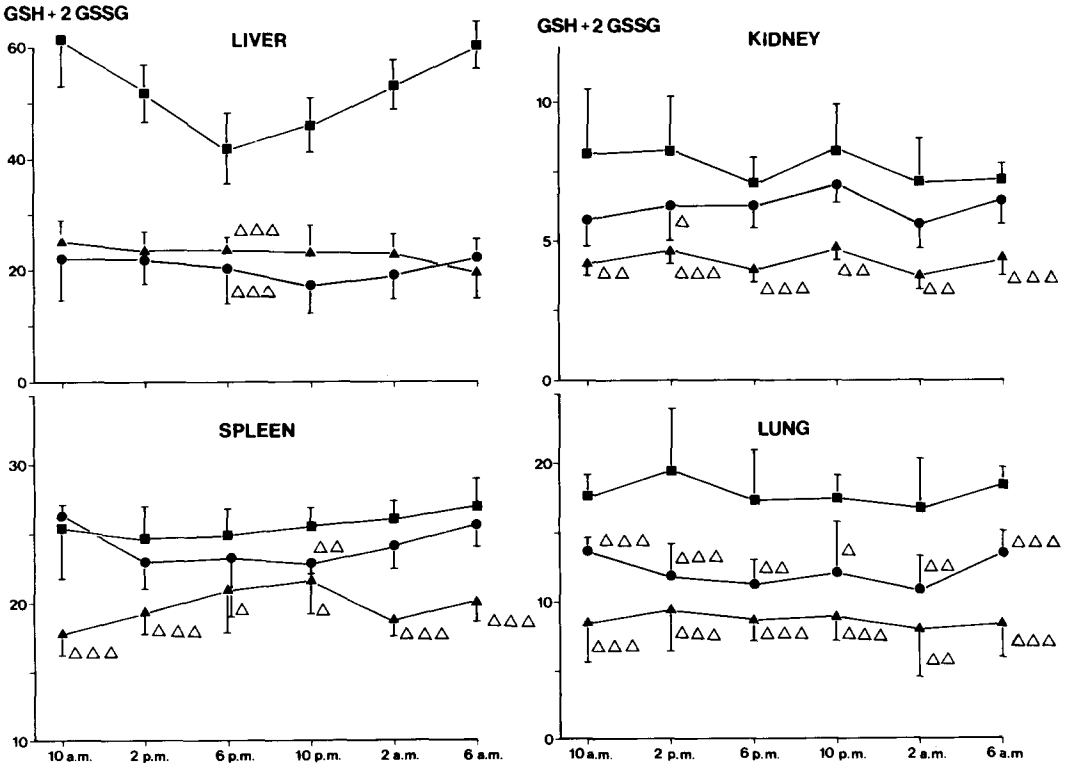

Fig. 1. Diurnal variation of the reduced plus oxidized glutathione content of various mouse organs under different nutritional conditions: ■, fed; ●, fasted for 24 hr; ▲, fasted for 48 hr. Data (mean ± S.D.) are given in nmole per mg organ protein. N = 5. △ $P \leq 0.05$; △△ $P \leq 0.01$; △△△ $P \leq 0.001$ with respect to fed state (upper curves). Within the diurnal glutathione profile at a given nutritional state, the data for liver were significantly different between 6 a.m. and 6 p.m. ($P \leq 0.001$). All other profiles showed no internal significant differences. The protein content of the liver was also subject to starvation. It was 170 ± 17 mg per g wet weight in fed animals, 157 ± 15 after 24 hr, and 149 ± 21 after 48 hr of starvation (cf. Fig. 2, where the results are expressed per gram liver wet weight).

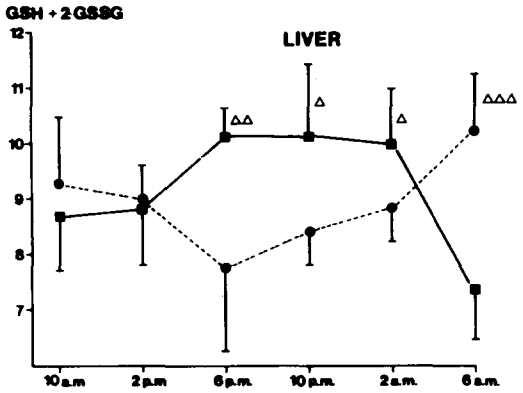

Fig. 2. Diurnal variation of the hepatic glutathione content of mouse liver during free access to food (dashed line) and an artificially reversed feeding (solid line). The animals were fed from 7 a.m. to 5 p.m. while food was withdrawn during the night. Data (mean ± S.D.) are given in μmol glutathione per gram liver wet weight. N = 7–9. The statistical significance between the glutathione content at 6 a.m. vs 6 p.m. was $P \leq 0.001$ in the normally fed group, and $P \leq 0.001$ in the reversed fed group. For significances see legend to Fig. 1.

trates that this regimen results in a reversal of the hepatic diurnal glutathione fluctuation.

In a previous paper, we showed that a single intravenous injection of 16.2 μmol glutathione or liposomally entrapped glutathione restored the liver glutathione content of starved animals within 2 hr to the level observed in fed animals [7]. The animals that had received liposomal GSH were fully protected against paracetamol-induced liver necrosis while the animals which were injected with GSH solution were much less protected. Since either treatment resulted in a similar liver GSH content of 44 nmole/mg protein, the biological potency of liver GSH is very different and depends on the form of administration. Therefore we were interested to learn whether this apparently well-regulated upper level of the glutathione content could be influenced by injection of GSH at the minimum or maximum of its natural diurnal rhythm.

Table 1. Influence of different forms of GSH injection on mouse liver organ glutathione content

		Untreated	GSH solution	GSH liposomes
Liver	M	25.0 ± 3.9	35.2 ± 4.6*	36.5 ± 3.1†
	E	23.6 ± 1.8	41.9 ± 2.9†	41.0 ± 4.6†
Kidney	M	4.2 ± 0.3	4.4 ± 1.2	3.9 ± 1.3
	E	4.0 ± 0.4	5.9 ± 0.4	6.6 ± 0.9
Spleen	M	11.1 ± 1.5	11.6 ± 1.6	59.6 ± 4.8†
	E	10.0 ± 1.2	8.4 ± 0.6	57.4 ± 3.3†
Lung	M	8.6 ± 3.0	8.1 ± 1.4	8.3 ± 0.9
	E	8.7 ± 1.4	9.6 ± 1	10.7 ± 2.3
Intestine	M	13.8 ± 1.9	11.1 ± 2	10.1 ± 0.7
	E	12.7 ± 2.1	9.4 ± 1.2	11.0 ± 0.9

Animals were maintained for 48 hr on a sucrose diet. A dose of 0.57 mmoles of GSH per kg was intravenously injected at 8 to 9 a.m. (M) or 6 to 7 p.m. (E). The organs were removed 2 hr later. N = 6–7. Data (mean ± S.D.) are given in nmol GSH-equivalents per mg organ protein.
* $P \leq 0.01$.
† $P \leq 0.001$.

The results in Table 1 show that injection of soluble GSH led only in the liver to a significant enhancement of the glutathione content, while the application of GSH encapsulated in liposomes increased the liver and spleen glutathione levels. The final concentration reached depended neither on the form of application nor on the time of administration to animals which had been at a minimal glutathione level after feeding sucrose for 48 hr. In the other organs investigated no prominently significant effect of any form of injected GSH was observed, irrespective of the time of administration.

This observation led us to investigate whether any other pharmacologically active sulfhydryl donor would be available for incorporation into glutathione in various organs. Table 2 contains the results of a series of experiments in which sulfhydryl drugs or

Table 2. Changes in glutathione content of various mouse organs after treatment of the animals with various sulfhydryl containing drugs or biological sulphur compounds

	Liver	Kidney	Spleen	Lung
Controls	23.0 ± 4.6	7.0 ± 1.5	12.7 ± 0.9	12.6 ± 2.3
BAL	18.3 ± 3.5	10.4 ± 3.1	13.1 ± 1.0	10.1 ± 0.9
Mesna	17.6 ± 3.9	6.8 ± 0.7	12.2 ± 0.9	11.2 ± 1.2
Thiola	18.3 ± 2.9	9.4 ± 1.5	12.5 ± 2.0	9.9 ± 1.5
D-Penicillamine	21.2 ± 4.2	9.0 ± 3.7	12.3 ± 0.9	12.6 ± 2.3
Cysteamine	28.0 ± 2.9	7.5 ± 1.5	12.4 ± 0.8	13.6 ± 3.4
N-Acetylcysteine	34.1 ± 1.5†	5.8 ± 1.7	12.3 ± 0.3	13.5 ± 1.9
L-Methionine	39.7 ± 1.7†	10.3 ± 0.9*	12.9 ± 1.0	12.0 ± 1.4
GSH	41.8 ± 6.3†	8.3 ± 2.9	12.5 ± 0.7	12.8 ± 1.8
GSSG	38.9 ± 5.4†	8.3 ± 1.5	13.0 ± 1.0	15.0 ± 2.5

Animals were maintained on a sucrose diet for 48 hr. The compounds or saline were intravenously injected at 6 p.m. 2 hr before removal of organs at a dose of 0.5 mmole per kg. N = 5. Data (mean ± S.D.) in nmol GSH + 2 GSSG per mg protein.
BAL = 2,3-dimercaptopropanol; Mesna = 2-mercaptoethanesulfonic-acid, Thiola = *N*-2-mercaptopropionylglycine.
* $P \leq 0.01$.
† $P \leq 0.001$.

biological sulphur compounds were injected into animals with a lowered glutathione content in the dayti. ıe, i.e. the minimum in the diurnal rhythm. The data show that almost only in the liver is an enhancement of the organ glutathione content achieved 2 hr after injection. Equimolar doses of *N*-acetylcysteine and oxidized glutathione showed comparable potencies for enhancing intrahepatic glutathione. Soluble GSH as well as methionine had a statistically significantly greater effect than *N*-acetylcysteine (GSH $P \leq 0.05$; methionine $P \leq 0.001$). None of these compounds affected the glutathione content of kidney, spleen or lung. With all other drugs no significant effect on GSH + 2 GSSG levels in the four organs investigated was observed. This indicates that the pharmacological activities of these compounds are not related to the glutathione system.

DISCUSSION

This study confirms the existence of a diurnal hepatic glutathione rhythm in rodents [2–5, 16]. It extends these findings by several additional observations: firstly, our results show that the diurnal variation of the liver glutathione content is abolished during starvation or a food intake lacking dietary protein; secondly, the demonstration of reversal of the rhythm by an artificially reversed feeding cycle provides a conclusive rationale for regarding the precursor intake as the cause of this phenomenon; thirdly, the results show that in addition of the kidney, the lungs as well as the spleen glutathione contents decrease at least twofold upon starvation. The toxicological relevance of this biochemical observation on inhalation or systematic toxicology remains to be studied. For mouse liver, an inverse correlation between the actual liver glutathione content and the extent of paracetamol toxicity was clearly shown [4]. It has been also demonstrated in rat liver, that the decrease of liver glutathione induced by starvation was not accompanied by a change in the organs GSSG content [17]. In our own study, we measured also the liver's content of protein-bound mixed disulfides. It was 30 ± 14 nmole per gram liver or 0.2 ± 0.07 nmole per mg protein, independent of the animals' nutritional state. Therefore we believe that this very small part of liver glutathione plays no role in this context.

It seemed feasible that at a minimum evening glutathione content the rodent liver would accept a glutathione supplement more readily in order to increase the organ content of the tripeptide than in the morning when the physiological maximum is achieved; (even when the rhythm is masked by starvation). This expectation did not hold true, i.e. the time of administration of free soluble or liposomally entrapped GSH did not significantly influence the final upper glutathione level reached 2 hr after treatment. Incidentally, this upper limit also corresponded to the minimum reached physiologically in the evening during the fed state (i.e. 42 ± 7 nmole/mg), while the physiological morning level (i.e. 62 ± 8 nmole/mg) was never reached by any treatment. In analogy to the well-known diurnal zonal variation of the hepatic glycogen content one could postulate that morphologically different areas of a liver acinus contain not only varying amounts of glutathione at different times of the day but also can be differentially reached by the tripeptide imported from the extracellular space. Indeed, a glutathione gradient within the liver lobule has been described [14]. We conclude from this series of experiments that it is not the regulation of glutathione biosynthesis or degradation on an enzymatic level that causes diurnal variations of the compound but simply the alimentary availability of precursors.

Therefore, it seemed logical to study whether other sulphur compounds could possibly also act as precursors. The results of this availability study indicate that several different radioprotective, anti-inflammatory or antineoplastic sulphur-containing drugs failed to show any consistent influence on organ glutathione contents. This finding suggests that their protective effect is mediated by a mechanism unrelated to the glutathione system.

In this respect, *N*-acetylcysteine seems to be an exception being able to enhance the glutathione content only in the liver.

This hepatic availability of *N*-acetylcysteine had already been studied in detail in animals and man and has been evoked as a rationale for the protective effect of the compound against drug induced liver damage [9]. An optimum supply for the liver, however, seems to be guaranteed by administration of methionine which is known to be used for glutathione synthesis via the cystathionine pathway [15]. Since GSH or GSSG injected in free forms are very rapidly degraded renally and redistributed after tubular reabsorption of the amino acids, administration of the tripeptide as such practically results in a delayed cysteine supply to the liver which should lead to the same effects as giving methionine. This was indeed observed. It is pointed out, however, that the biological protective potency of intrahepatic glutathione raised by different means is likely to be very different. We showed for example, that soluble GSH or *N*-acetylcysteine treatment of mice protects mice only marginally against paracetamol-induced liver necrosis, while liposomally entrapped GSH leading to similar liver glutathione contents resulted in an optimal preventive protection against high doses of the drug [10]. Together with the results of this study it would seem promising to investigate the biological potency of various forms of glutathione under different nutritional (i.e. depending on the diurnal rhythm) and pharmacological influences.

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