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# Glutathione deficiency leads to mitochondrial damage in brain

**(buthionine sulfoximine/glutathione ester/turnover/hydrogen peroxide/animal model)**

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**ABSTRACT** **Glutathione deficiency induced in newborn rats by giving buthionine sulfoximine, a selective inhibitor of γ-glutamylcysteine synthetase, led to markedly decreased cerebral cortex glutathione levels and striking enlargement and degeneration of the mitochondria. These effects were prevented by giving glutathione monoethyl ester, which relieved the glutathione deficiency, but such effects were not prevented by giving glutathione, indicating that glutathione is not appreciably taken up by the cerebral cortex. Some of the oxygen used by mitochondria is known to be converted to hydrogen peroxide. We suggest that in glutathione deficiency, hydrogen peroxide accumulates and damages mitochondria. Glutathione, thus, has an essential function in mitochondria under normal physiological conditions. Observations on turnover and utilization of brain glutathione in newborn, preweaning, and adult rats show that (*i*) some glutathione turns over rapidly ($t_{1/2}$, ≈30 min in adults, ≈8 min in newborns), (*ii*) several pools of glutathione probably exist, and (*iii*) brain utilizes plasma glutathione, probably by γ-glutamyl transpeptidase-initiated pathways that account for some, but not all, of the turnover; thus, there is recovery or transport of cysteine moieties. These studies provide an animal model for the human diseases involving glutathione deficiency and are relevant to oxidative phenomena that occur in the newborn.**

Studies in which glutathione (GSH) deficiency was induced in animals by administering L-buthionine (*S,R*)-sulfoximine (BSO) (1, 2), a transition-state inhibitor of γ-glutamylcysteine synthetase (3, 4), showed that GSH deficiency leads to myofiber degeneration in skeletal muscle (5), damage to type 2-cell lamellar bodies and capillary endothelial cells in the lung (6), and epithelial-cell damage to jejunum and colon (7) in adult mice, and to lens epithelial-cell degeneration and cataract formation in newborn mice (8, 9) and rats (9). These effects, which were invariably accompanied by markedly decreased mitochondrial GSH levels, were associated with mitochondrial swelling with vacuolization and rupture of cristae and mitochondrial membranes as seen by EM. The isolated mitochondria exhibited decreased citrate synthase activity.

It should be emphasized that these effects occurred without application of stress (e.g., increased oxygen, drugs, radiation) and that they were completely prevented by administration of GSH monoesters (10–13). In the absence of evidence that BSO itself exerts a separate type of toxicity other than its effect on the enzyme that catalyzes the first step of GSH synthesis, it may be concluded that a major effect of GSH deficiency is mitochondrial damage. Although mitochondria have long been known to contain GSH, only recently was mitochondrial GSH found to originate from the cytosol and to be imported into mitochondria by a system that contains a high-affinity transporter (14, 15). Not all oxygen used by mitochondria is reduced to water, but a significant fraction of it is converted, apparently through superoxide, to hydrogen peroxide (16–19), which, as indicated by recent studies in this laboratory (5–7, 9), produces extensive mitochondrial damage when GSH levels are greatly decreased. These findings thus demonstrate an important function of GSH, which is further elucidated here in brain studies.

The turnover of brain GSH has been thought to be relatively slow compared with that of kidney and liver; thus, a $t_{1/2}$ value of 70 hr was estimated for rat brain (20) as compared, for example, with a $t_{1/2}$ value of ≈29 min for mouse kidney (21). Tissue GSH turnover rates have been estimated from the rates of decline of GSH levels after BSO administration (2, 5–7, 9). Because BSO is poorly transported across the blood–brain barrier (2, 22, 23), this approach is less useful for brain. However, BSO ethyl ester is readily transported into the brain, and studies with this compound provided evidence for a fraction of cerebral cortex GSH that rapidly turns over in adult mice (23).

In these studies, BSO was administered to newborn rats, which lack a fully developed blood–brain barrier (22); BSO was given to adult rats in 15% dimethyl sulfoxide, which increases BSO uptake by brain (23). These approaches substantially decreased brain GSH levels. The present studies provide an animal model for the human diseases in which marked neurological changes are associated with inborn deficiency of either GSH synthetase or γ-glutamylcysteine synthetase (24). The findings are also relevant to GSH metabolism and to oxidative phenomena that occur in the newborn.

## EXPERIMENTAL PROCEDURES

**Materials.** Sprague–Dawley rats (female, timed pregnant; both sexes, newborn to 3 weeks old; male, 14 weeks old) were obtained from Taconic Farms and given laboratory chow and drinking water ad libitum. Newborn rats breast feed until ≈18 days of age.

BSO (3, 4, 25) and GSH monoethyl (glycyl) ester semihydrosulfate (12, 13) were prepared as described. Solutions of GSH ester were carefully adjusted to pH 6.5–6.8 by adding 2 mM sodium hydroxide immediately before use. BSO and GSH ester were given in doses specified below in isosmolar solutions; in some experiments BSO was given in 15% (vol/vol) dimethyl sulfoxide (23). In control experiments rats were given equivalent amounts of GSH, ethanol, and sodium sulfate.

**Methods.** Samples of brain were obtained after anesthetizing the rats by i.p. injection of xylazine (4–6 mg/kg) and ketamine (40–60 mg/kg). After the peritoneal and thoracic cavities were opened (2 min) (6, 9), the brain was perfused for 1 min with 10–15 ml of cold saline through the left ventricle after the right ventricle had been opened. After the skull was entered, a portion of parietal cortex was excised, freed of its meninges, rinsed in cold saline, blotted, weighed, and ho-

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Abbreviations: GSH, glutathione; BSO, L-buthionine (*S,R*)-sulfoximine; GSSG, glutathione disulfide.

mogenized in 5 vol of 5% (wt/vol) sulfosalicylic acid. The homogenate was then centrifuged for 2 min at 10,000 × *g* at 4°C, and the supernatant was immediately analyzed for GSH (26).

Blood was drawn from the common carotid artery and the internal jugular vein in lightly anesthetized (one-third of the anesthetic doses given above) rats after careful exposure of the vessels. Blood (0.3–0.4 ml) samples were slowly aspirated through a 26-gauge heparinized needle into a tuberculin syringe from the internal jugular vein and then from the common carotid artery. The saggittal sinus was sampled after incising the skin and dissecting the fascia over the lambdoid suture; a 23-gauge butterfly needle was used for aspiration. Blood samples were mixed with EDTA (final concentration, 3 mM) and immediately centrifuged for 1 min at 10,000 × *g*. A portion of plasma (0.1–0.2 ml) was mixed with 0.1 vol of 43% (vol/vol) sulfosalicylic acid and centrifuged at 10,000 × *g* for 2 min at 4°C; the supernatant solution was used immediately for GSH determinations (26).

Total GSH [GSH plus glutathione disulfide (GSSG)] was determined by the GSSG reductase-5,5-dithiobis-2-nitrobenzoate recycling method (26–28). GSSG was estimated by the 2-vinylpyridine procedure (29). GSH was also determined after reduction with 5 mM dithiothreitol (26, 30); this determination includes GSH, GSSG, and GSH mixed disulfides. Blood contamination (31) accounted for <1% of the GSH found in brain (32).

Citrate synthase was determined (33, 34) on mitochondria isolated (35, 36) from cerebral cortex. Tissue samples were homogenized in 10 vol of 5 mM Tris·HCl, pH 7.4/70 mM sucrose/220 mM mannitol/0.1% albumin/0.1 mM sodium EDTA with a Dounce homogenizer (10 strokes) (9). Preparations containing mitochondria were obtained by centrifugation at 800 × *g* and 10,000 × *g*, each for 10 min at 4°C, and enzyme activity was measured after freezing and thawing. GSH was determined after treatment with sulfosalicylic acid. Purity of the mitochondrial preparations, assessed by EM, revealed, as expected (36), ≈20% contamination by synaptosomes.

EM of cerebral cortex was performed on 12-day-old rats that were anesthetized and perfused as described above. Initial perfusion (≈2 sec) was through the aorta with 100 mM sodium phosphate buffer, pH 7.4, containing sufficient NaCl to yield 300 mosM; 50 ml of 4% (wt/vol) glutaraldehyde/4% (wt/vol) paraformaldehyde in sodium phosphate buffer was perfused at 80–90 mm Hg (1 mm Hg = 133 Pa) for 10 min. After perfusion, the tissue remained *in situ* for 1 hr at 26°C. The brain was then excised and placed in glutaraldehyde/paraformaldehyde for 2 hr at 26°C. Sections of cortex were washed with sodium phosphate buffer at 4°C, fixed in 1% $O_8O_4$, dehydrated in ethanol and propylene oxide, and embedded in Epon 812 (5).

## RESULTS

**Effect of BSO on GSH Levels in the Cerebral Cortex.** Administration of BSO to adult mice or rats leads to a relatively small decrease in the level of GSH in the brain; thus, in the initial *in vivo* studies on BSO (2) the GSH level of the brain of mice examined 2 hr after a single BSO dose decreased ≈7%; after BSO was given orally for 15 days the decline was ≈16%. Subsequent studies have yielded similar results on adult mice, but a substantial decrease was found in the GSH levels of brain of newborn mice, an effect that could be ascribed to an undeveloped blood–brain barrier (22). A similar effect has been seen in the eye; thus, BSO administration leads to formation of cataracts in newborn mice and rats but not in older animals (8, 9). In another approach, BSO was administered directly to the brain by means of an indwelling catheter implanted in the lateral ventricles (37, 38).

In the present studies, administration of a single BSO dose to adult rats led to decline of cortical GSH at an initial rate of ≈1.5% per min (Fig. 1 *Inset*, curve 3). Repeated BSO doses led to further decline in the GSH level in the cortex, which reached ≈60% of control value in 10 days (Fig. 1, curve 3). Administration of BSO to preweaning rats (14–16 days of age; ≈35 g) caused a greater decline in cortical GSH than observed in adults (Fig. 1, curve 2). In preweaning rats, the GSH level decreased to ≈30% of control value after 2 days and then increased as the animals became older and presumably developed a blood–brain barrier. In adult and preweaning rats, the decline rate of GSH levels decreased significantly with time after BSO administration. These rates (expressed as $t_{1/2}$ values) may be estimated, but because several factors may influence the decline rate of GSH levels, these values must be considered as tentative. For adult rats, $t_{1/2}$ values of ≈30 min, 60 hr, and 40 days may be extracted from the data.

Treatment of newborn rats with BSO leads to a very rapid decline in the cortical GSH level; tentative estimates of the decline rates for newborn animals are ($t_{1/2}$) 8 min, 60 hr, 160 days. The level of cortical GSH increases in the newborn animals as they age, after ≈10 days. About 40% of newborn rats given this BSO dose do not survive for longer than ≈3 days. [BSO is even more toxic when given at birth (9); in the present studies BSO was given 36–48 hr after birth.] The BSO-treated newborn rats exhibited definite signs of neurological disturbance, including lethargy, intermittent tremors (accentuated by perceptual and physical stimuli), and minor fits. Only the rats that continued to breast feed survived and were used in this study.

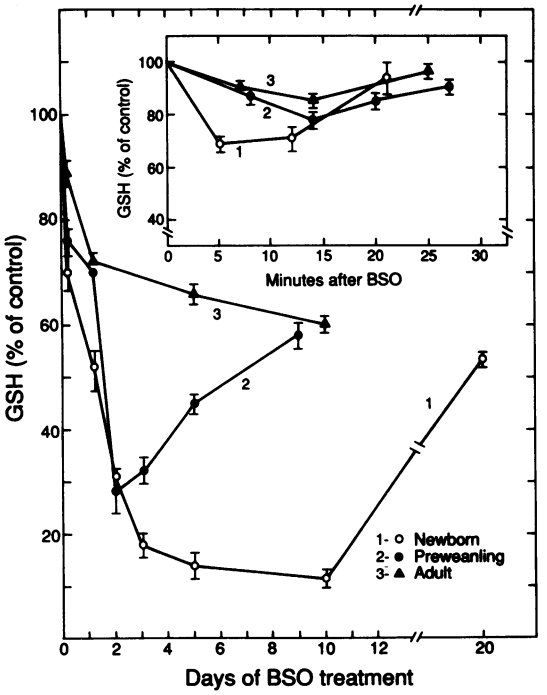


FIG. 1. Effect of BSO administration on cerebral cortex GSH levels. Newborn rats (7.5–8.0 g) were injected with BSO (2 mmol/kg) i.p. twice daily at 9 a.m. and 9 p.m. Preweaning rats (34–36 g) were given two daily doses of BSO (3 mmol/kg). Adult rats (400–500 g) were given two daily doses of BSO of 4 mmol/kg in 15% dimethyl sulfoxide (23). GSH levels were determined in the cerebral cortex initially and after BSO injection. (*Inset*) Values after a single dose of BSO (2–4 mmol/kg); adult rats were injected with BSO in 15% dimethyl sulfoxide. Control values were 1.36 ± 0.08, 1.27 ± 0.07, and 1.22 ± 0.5 μM for newborn, preweaning, and adult rats, respectively. All determinations were done in duplicate on samples from three to six animals. Values are means ± SD.

Table 1. Effects of BSO, GSH, and GSH ester on GSH levels of cerebral cortex of newborn rats

| Treatment | Experiment | GSH | |
|---|---|---|---|
| | | Cortex, μmol/g | Mitochondria, nmol/mg of protein |
| 3 days | | | |
| None (controls) | 1 | 1.01 ± 0.05 | 12.2 ± 0.75 |
| BSO | 2 | 0.19 ± 0.01 | 4.92 ± 0.22 |
| BSO + GSH | 3 | 0.22 ± 0.01 | 4.83 ± 0.08 |
| BSO + GSH ester | 4 | 0.31 ± 0.01 | 8.78 ± 0.41 |
| 9 days | | | |
| None (controls) | 5 | 1.12 ± 0.11 | 8.16 ± 0.83 |
| BSO | 6 | 0.13 ± 0.01 | 1.29 ± 0.11 |
| BSO + GSH | 7 | 0.14 ± 0.01 | 1.22 ± 0.09 |
| BSO + GSH ester | 8 | 0.38 ± 0.02 | 7.55 ± 0.68 |

GSH (GSH + GSSG) levels (26) are given as means ± SD ($n$ = 3–5). Newborn rats (36–48 hr of age) were injected i.p. with saline or BSO (2 mmol/kg) twice daily for 3 or 9 days at 9 a.m. and 9 p.m. In experiments 4 and 8, the rats were also given (twice daily at 11 a.m. and 11 p.m.) GSH monoethyl ester (2.5 mmol/kg). In experiments 3 and 7, the rats were also given an isosmolar (pH 6.5–6.8) solution containing GSH (2.5 mmol/kg), ethanol, and $Na_2SO_4$ (in amounts equivalent to those of GSH ester given in experiments 4 and 8). Equal volumes of saline were given in experiments 2 and 6 to match those given in experiments 3, 4, 7, and 8.

Data on the effects of administration of BSO to newborn rats on the GSH levels of cerebral cortex and the mitochondrial fraction obtained from this tissue are summarized in Table 1. After BSO administration for 3 days, the GSH level of the cerebral cortex decreased to ≈19% of untreated control levels; the mitochondrial GSH decreased to ≈40% of control level (experiment 1). Similar results were obtained 9 days after BSO treatment (experiment 6). Thus, the GSH level of the cerebral cortex declined to ≈13% of control values, whereas the level found in the mitochondrial fraction was ≈16% of control values. In experiment 3 of the table, GSH was administered with BSO for 3 days; there was no significant difference between levels of cortical GSH or of the mitochondrial fraction as compared with those of rats given BSO alone (experiment 2). However, when GSH ester was given together with BSO (experiment 4), the levels of GSH in the cortex and in the mitochondrial fraction were substantially higher than found after giving BSO alone (experiment 2). Studies conducted after 9 days of treatment also showed that administration of GSH together with BSO gave GSH levels about the same as found after giving BSO alone (compare experiments 7 and 6). On the other hand, when GSH ester was given together with BSO, the GSH level of the cortex was about twice that found in animals treated with BSO alone, and the level of GSH in the mitochondrial fraction was ≈6-fold higher than that found after treatment with BSO alone (compare experiments 8 and 6). Note that the mitochondrial level of GSH in the animals given BSO plus GSH ester (experiment 8) was, within experimental error, about the same as that of the untreated controls (experiment 5).‡

**Mitochondrial Damage in Cerebral Cortex After Administration of BSO to Newborn Rats.** The cerebral cortices of newborn rats given BSO, BSO plus GSH, and BSO plus GSH ester, as described in Table 1, were subjected to EM (Fig. 2). Fig. 2*a* shows a section from the cerebral cortex of a control (untreated) rat: many mitochondria are visible as the denser bodies throughout the section, which also reveals other structures of the cerebral cortex. In Fig. 2*b*, a section of the cerebral cortex of a BSO-treated animal, fewer mitochondria appear, and the mitochondria present are strikingly enlarged as compared with the normal mitochondria shown in the control section. The internal structure of the mitochondria is apparently destroyed. That the mitochondria are increased to at least several times the size of normal cerebral cortex mitochondria is especially notable. The remarkable mitochondrial swelling and degeneration seen after BSO treatment are also found in the cerebral cortex obtained from animals treated with both BSO and GSH (Fig. 2*c*). On the other hand, sections of cerebral cortex obtained from newborn rats treated with BSO plus GSH ester (Fig. 2*d*) showed many mitochondria of normal size and were indistinguishable from sections of normal cortex.

Additional assessment of mitochondrial damage was obtained by determinations of citrate synthase [a mitochondrial matrix marker enzyme (33)] after 9 days of BSO administration. The citrate synthase activity was 0.66 ± 0.01 mmol/min per mg of protein ($n$ = 3) compared with control values of 0.87 ± 0.01 mmol/min per mg of protein ($n$ = 3).

**Disappearance of Plasma GSH on Passage Through Brain.** Brain GSH presumably arises, as it does in other tissues, by synthesis from its constituent amino acids (1, 39, 40). Cysteine, frequently the limiting amino acid for GSH synthesis, is present in the plasma and is transported to cells including those of brain. Plasma GSH also serves in supply of cysteine moieties to the tissues. The liver provides most of the plasma GSH that is utilized by several tissues of which the kidney is of major significance. Previous studies on rats indicated that about two-thirds of the plasma GSH is utilized on passage through the kidney (41, 42). The lung also utilizes plasma GSH (6). Such utilization appears dependent upon γ-glutamyl transpeptidase, which catalyzes the initial step in the degradation of GSH. The kidney has the highest levels of γ-glutamyl transpeptidase, but the enzyme is also found in virtually all other tissues, including the brain (43). We found values for cerebral cortex γ-glutamyl transpeptidase [determined as described (43)] for 1-day-old, 14-day-old, and adult rats of 52.6 ± 6.0, 68.6 ± 8.0, and 155 ± 8.1 nmol/hr per mg of protein, respectively.

We examined the plasma GSH level in blood obtained from the carotid artery, the internal jugular vein, and the saggittal sinus of adult and preweaning rats and found a significant arteriovenous difference across the brain (Table 2). The values for jugular vein and saggittal sinus plasma GSH are similar; the utilization of plasma GSH in the cerebral cortex therefore appears to be similar to that of the average of whole brain. Cerebral blood flow in the adult rat has been estimated at 100 ml/min per 100 g of brain (44–46); a value of 129 ml/min per 100 g of brain was estimated for preweaning rats (47). These values, together with those given in Table 2, may be used to estimate the utilization of plasma GSH by the cerebral cortex; values of ≈3.4 and 4.2 nmol/min per g are found for adult and preweaning rats, respectively. These values are much lower than those for the initial rate of turnover of cerebral cortex GSH, estimated from the initial rate of GSH decline after BSO, which are ≈15–25 nmol/min per g. Therefore, utilization of plasma GSH alone does not appear to account for the cysteine requirement for GSH turnover. Cysteine moieties may be recovered during the turnover process (1) or may be transported as cysteine or other cysteine-containing molecules in plasma. Cysteine has been reported to be transported across the blood–brain barrier (48). Cystine, also present in plasma, might be transported as γ-glutamylcystine after formation of this compound by γ-glutamyl transpeptidase (49, 50) or as cystinyl bisglycine.

‡Several of the newborn rats that were treated with GSH ester died. As noted previously (9), administration of GSH ester to newborn rats is accompanied by some mortality, which we tentatively ascribe to the toxicity of the alcohol released; similar mortality was found after giving equivalent doses of alcohol. Such toxicity was not observed in older rats or mice.

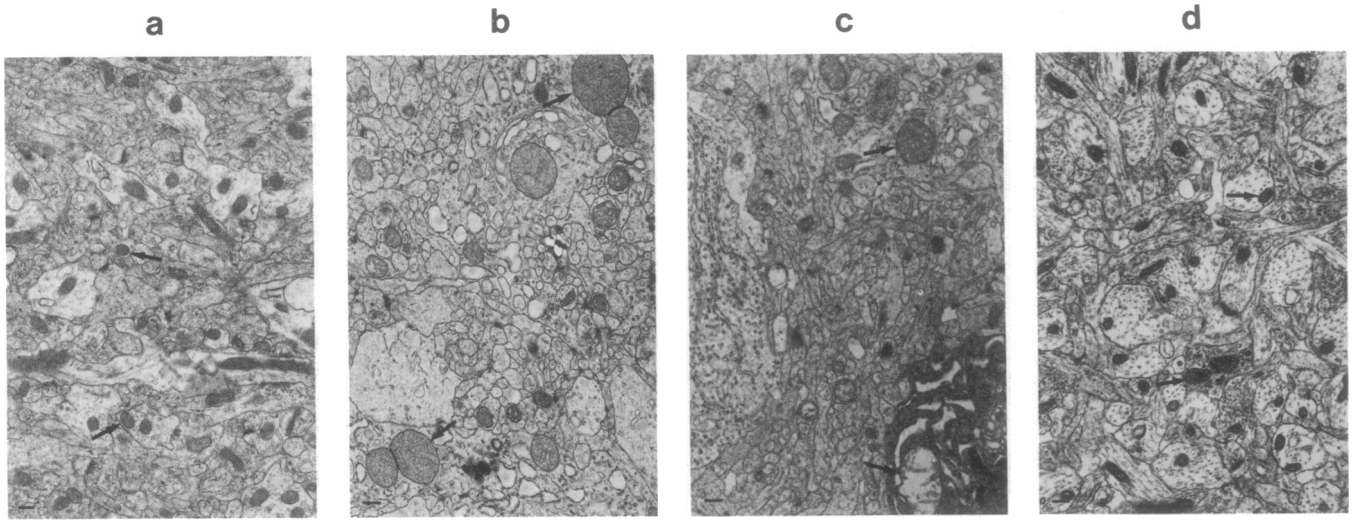


FIG. 2. Mitochondrial damage in cerebral cortex of newborn rats after BSO administration. The rats were given saline (*a*), BSO (*b*), BSO + GSH (*c*), or BSO + GSH ester (*d*) as stated in Table 1 from 48 hr to 11 days of age. In *b* and *c* there is marked mitochondrial swelling and degeneration (see text) that is not seen in controls (*a*) or GSH ester-treated animals (*d*). In *a–d*, two representative mitochondria are indicated by arrows. (×6600; bar in lower left-hand corner = 0.37 μm.)

Utilization of plasma GSH by brain probably occurs by a pathway analogous to that previously shown for kidney and other tissues (1, 39, 40) involving extracellular or membranous breakdown of GSH followed by uptake of products (amino acids, dipeptides) and intracellular GSH synthesis. Although cerebral cortex and other regions of the brain have substantial levels of γ-glutamyl transpeptidase, the possibility that intact GSH might be transported into brain needs to be considered. Previous studies did not reveal transport of GSH (51). A recent report gives data that were interpreted to show carrier-mediated transport across the blood–brain barrier in the rat; uptake was reported as saturable with an apparent $K_m$ value of 5.84 mM (52). The data presented do not provide a value for uptake at physiological levels of plasma GSH (≈22–27 μM) (53). Although acivicin (an inhibitor of γ-glutamyl transpeptidase) was administered (52), γ-glutamyl transpeptidase would not be predicted to be completely inhibited under these conditions (54). Consistent with this interpretation, 17% of the GSH was reported to be metabolized (52). Although doubt thus exists as to whether (and if so how much) intact GSH might have been transported in these studies, should such a transport system exist [with $K_m$ of 5.84 mM (52)], increasing plasma GSH levels would be predicted to increase brain GSH levels by direct GSH transport. However, in our studies (Table 1) we found no significant increase of the cortical GSH levels after substantial GSH doses; under such conditions, plasma GSH levels increase to values of 5–25 mM in adult rats (6), and we found similar levels in newborn rats treated with GSH. Furthermore, administration of GSH did not prevent mitochondrial swelling and damage (Fig. 2), whereas giving GSH ester was fully protective. Thus, the findings do not seem to provide evidence for quantitatively significant transport of intact GSH into the cerebral cortex, even after substantial (200- to 1000-fold) increase of the plasma GSH level. Despite these considerations, which relate to the mechanism of GSH uptake, there can be little doubt that GSH administration (which serves as a good source of cysteine moieties) may have value in increasing GSH levels of brain (and other tissues), as has been discussed (7).

Table 2. Plasma GSH levels

| | Carotid, μM | Jugular (saggittal sinus), μM | A − V, μM |
|---|---|---|---|
| Adult | | | |
| GSH | 14.7 ± 0.9 | 8.92 ± 0.7 (8.99 ± 0.5) | 5.8 (5.7) |
| GSSG | 1.3 ± 0.5 | 1.3 ± 0.5 (1.4 ± 0.2) | |
| GSH (after dithiothreitol) | 20.8 ± 1.0 | 16.5 ± 0.2 (17.0 ± 0.6) | 4.3 (3.8) |
| Preweaning | | | |
| GSH | 18.9 ± 1.3 | 12.3 ± 0.6 (13.6 ± 0.8) | 6.6 (5.3) |
| GSSG | 2.6 ± 0.2 | 2.3 ± 0.4 (2.4 ± 0.4) | |
| GSH (after dithiothreitol) | 27.1 ± 2.1 | 20.5 ± 2.0 | 6.6 |

GSH, GSSG, and GSH (after treatment of samples with dithiothreitol) were determined as described in text; values are given as means ± SD ($n$ = 3–5). A − V, differences between carotid artery plasma values and those of plasma drawn from the jugular vein (or saggittal sinus).

## DISCUSSION

It has long been known that mitochondria produce hydrogen peroxide (16–19). Several possible pathways can be invoked for disposal of hydrogen peroxide (19). However, GSH-dependent reactions are clearly of major importance for reducing hydrogen peroxide in mitochondria. The present findings offer an experimental animal model for the human diseases in which GSH synthesis is deficient. Patients with these conditions typically have symptoms and signs of central nervous system dysfunction (24). The findings are also relevant to certain newborn humans, especially premature infants, who often exhibit a deficiency of transsulfuration (55), the process by which the sulfur atom of methionine is used for cysteine synthesis (56). Under these conditions, cysteine becomes an essential nutrient. The i.v. alimentation therapy commonly given to such patients does not usually include cysteine, so that they may become deficient in cysteine and thus in GSH. Inhibition of GSH synthesis in the newborn rat is frequently fatal, suggesting that GSH is of crucial metabolic importance; further studies on this and on therapies that increase tissue GSH levels in the newborn are necessary.

Brain GSH is probably compartmented, as it is for other metabolites (57, 58). The decline in GSH level of cerebral cortex after giving BSO occurs at substantially different rates, possibly reflecting several pools of GSH that may occur, for example, in mitochondria, neurons, and glial cells. However, until information is available about the rate of BSO entry into such structures and their other metabolic properties, definite conclusions cannot be drawn. Notably, cultured astrocytes from newborn mice have much higher GSH levels than do neurons (59). Further efforts are needed to elucidate the complexities of GSH metabolism and function in brain.

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