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Glutathione ester prevents buthionine sulfoximine-induced cataracts and lens epithelial cell damage

[mitochondria/glutathione mono(glycyl)ester/development/oxidative stress]

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ABSTRACT **Treatment of newborn rats and mice with buthionine sulfoximine, an inhibitor of glutathione synthesis, leads to development of cataracts, which are not prevented by treatment with glutathione, but they are prevented by treatment with glutathione monoester. Cataracts are associated with glutathione deficiency in the lens epithelium, which undergoes severe degeneration. The findings indicate that glutathione normally functions in the protection of the lens and lens epithelium against oxidative injury, suggesting that procedures that increase lens glutathione levels might be useful for prevention of other types of cataracts. Relatively low doses of buthionine sulfoximine produce cataracts in newborn animals, and treatment of pregnant mice with buthionine sulfoximine during the last part of gestation leads to cataract formation in the offspring. The high sensitivity of the developing lens to the effects of glutathione deficiency suggests that this tissue may be a useful model for studies on glutathione function.**

Glutathione (GSH) has several important functions in many tissues, one of which is protection against oxidative damage (1–3). The lens contains a relatively high level of GSH, and it is notable that many types of cataracts are associated with decreased levels of GSH in the lens (4–10). Administration of buthionine sulfoximine (BSO) (11), an effective inhibitor of γ-glutamylcysteine synthetase (the enzyme that catalyzes the first step in the biosynthesis of GSH), to mice and rats leads to markedly decreased levels of GSH in many tissues (12–14). Administration of BSO to mice 9–12 days old leads to low levels of lens GSH and to cataract formation (15). These and earlier findings suggest that there is a causal relationship between decreased levels of GSH and the formation of cataracts, but it is conceivable that the effect of BSO is mediated through another mechanism.

In the present work, we explored the effect of BSO on the lens in newborn mice and rats. Electron microscopic studies showed that administration of BSO causes severe degeneration of the lens epithelium. Epithelial damage and cataract formation are not prevented by simultaneous administration of GSH; however, administration of GSH monoester (L-γ-glutamyl-L-cysteinylglycyl isopropyl ester) prevents development of cataracts. Whereas the GSH molecule itself is not effectively transported into cells, GSH monoesters are transported and converted to GSH by intracellular hydrolysis (16–18). The present studies indicate that BSO-induced cataracts are produced by GSH deficiency and that GSH normally functions to protect the lens against oxidative injury, which is a major factor in cataract formation.

EXPERIMENTAL PROCEDURES

Materials. Sprague–Dawley rats and Swiss–Webster mice (timed pregnant) were obtained from Taconic Farms. L-Buthionine-(*S*,*R*)-sulfoximine (BSO) (11, 19, 20) and GSH monoisopropyl ester (16–18, 21, 22) were obtained as described. An aqueous solution of GSH monoisopropyl ester was prepared in the $\frac{1}{2}(H_2SO_4)$ form and adjusted to pH 6.5–6.8 by cautious addition of NaOH immediately prior to use. In studies in which this compound was used, control animals were given GSH, isopropanol, and Na_2SO_4.

Methods. The rats were injected intraperitoneally with the compounds described below. The animals were sacrificed by anesthesia with xylazine and ketamine (14) and were perfused through the left ventricle with 3 ml of cold saline for 1 min. The lenses were carefully dissected from the ciliary body and the lens capsule was pierced with a thin needle in the presence of 3 mM EDTA. For GSH analysis, two rat lenses were homogenized with 5 vol of 5% sulfosalicylic acid; after centrifugation, total GSH was determined by the enzymatic recycling method (23). Pregnant mice were treated with BSO by intraperitoneal injection (4 $mmol \cdot kg^{-1} \cdot day^{-1}$) and by adding BSO (20 mM) to the drinking water. The BSO level was determined as described (14).

Mitochondrial fractions of lenses were isolated from batches of 60 rat lenses (three separate batches per experiment) 74 hr after birth. The lenses were homogenized at 5°C by hand (10 strokes; Dounce homogenizer) in 5 vol of 5 mM Tris·HCl (pH 7.4) containing 220 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, and 0.1% bovine serum albumin, and were then subjected to differential centrifugation (24–26). Homogeneity of the mitochondrial preparations was checked by electron microscopy. Protein was determined as described (27) using bovine serum albumin as a standard.

For electron microscopy, the mice were perfused (for 2 min) through the left ventricle with 2 ml of 4% glutaraldehyde/4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) containing sufficient NaCl to bring the final mixture to 300 mosM. Perfusion of rats was carried out with 30 ml (for 10 min) at a pressure of 90 mmHg after opening the right ventricle by placing the tip of the needle in the ascending aorta and securing it with a silk suture.

RESULTS

Effects of BSO and GSH Ester on Lens GSH Levels and Cataract Formation. Rats were injected with BSO on the 2nd and 3rd days of life, and the levels of GSH in the lens were determined at intervals for 14 days. Four groups were used: (*i*) controls (untreated), (*ii*) rats given BSO only, (*iii*) rats given BSO plus GSH, and (*iv*) rats given BSO plus GSH ester (Fig. 1).

The levels of GSH in the lenses of control rats are higher at birth (≈8 μmol/g) than after 14 days or in older rats (4–5 μmol/g). The level of lens GSH decreases substantially on the 1st day of life [a marked decrease in GSH levels occurs

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Abbreviations: GSH, glutathione; BSO, buthionine sulfoximine.
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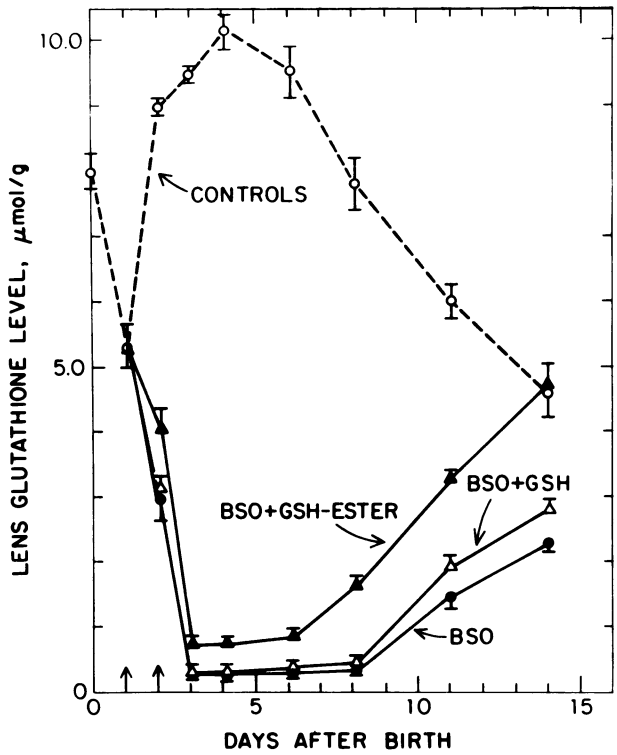

FIG. 1. Effect of BSO, GSH, and GSH monoester on lens GSH levels. Newborn rats (6 g) were injected intraperitoneally with saline (controls) or with BSO (3 mmol/kg) 26 and 50 hr after birth (arrows) and, as indicated, twice daily (10 a.m. and 10 p.m.) with GSH (isosmolar, adjusted to pH 6.8 with NaOH; 5 mmol/kg) plus isopropanol at 5 mmol/kg and Na_2SO_4 at 2.5 mmol/kg, or with GSH monoisopropyl ester·½(H_2SO_4) (isosmolar, adjusted to pH 6.8 with NaOH; 5 mmol/kg). Rats were sacrificed at the times indicated and their lenses were analyzed for GSH. The lenses of controls weighed 3.5, 6.0, 8.6, and 14 mg, respectively, at days 0, 4, 8, and 14. The corresponding weights were 3.5, 4.5, 5.0–5.2, and 8.5–9.5 mg, for the BSO (and BSO plus GSH)-treated rats, and 3.5, 5.8, 8.3, and 14 mg for the BSO plus GSH monoester-treated rats. Data are given as means ± SD (error bars) (n = 3–5). The number of cataracts found when rats treated in the same way spontaneously opened their eyes is recorded in Table 1. The controls and rats treated with BSO and GSH monoester opened their eyes on day 14; the other rats opened their eyes on day 15.

in most tissues of the newborn rat on the first day of life (unpublished data)] and then increases, reaching a maximum at ≈4 days, followed by a decrease to levels similar to those found in adults (Fig. 1). The lenses of newborn rats treated with BSO (on days 2 and 3) showed a marked decrease, to ≈0.2 μmol of GSH per g. This low level persisted for several days and then increased slowly. Treatment with BSO plus GSH (2 daily doses of 5 mmol/kg) gave virtually the same results (on days 3–8) as found after treatment of rats with BSO alone. On the other hand, the lenses of rats treated with BSO plus GSH monoester had ≈3-fold higher levels of GSH on days 3–6 (≈0.7 μmol/g), and the GSH levels increased markedly after day 6, reaching the control level by day 14.

All of the rats treated with BSO, as well as those treated with BSO plus isopropanol (or ethanol), and with BSO plus GSH (total of 53 animals) developed cataracts. None of the 31 control (untreated) rats or the 20 rats treated with BSO plus GSH monoester developed cataracts (Table 1). The dose of BSO given in these experiments (two injections of 3 mmol/kg on days 2 and 3) is close to that minimally required to produce cataracts. Thus, when rats were given only a single dose of BSO at 3 mmol/kg (on either day 2 or day 3), only 50–70% developed cataracts. When only a single daily dose of GSH monoester (5 mmol/kg) was given to BSO-treated rats, ≈50% of the rats developed cataracts.

Treatment with BSO led to a substantial decline in the level of lens mitochondrial GSH; simultaneous administration of GSH did not affect mitochondrial GSH levels significantly, whereas administration of GSH monoester led to higher levels (Table 1). The yield of mitochondrial protein from the lenses of BSO-treated rats (per unit weight of lens) was ≈50% that obtained from the controls; after treatment with BSO and GSH monoester, the yield was ≈70% that of controls. Determinations (on day 4) of citrate synthase, a mitochondrial marker (24–26), revealed an ≈75% decrease in activity in the mitochondrial fractions from BSO-treated rats [0.015 ± 0.003 (n = 3) μmol/min per mg of protein], as compared to the controls [0.061 ± 0.011 (n = 3) μmol/min per mg of protein]. Citrate synthase decreased only ≈30% after treatment with BSO and GSH monoester. These findings are in accord with the electron microscope studies, which showed extensive mitochondrial degeneration after treatment with BSO.

Electron Microscopy. Examination of the lenses of newborn rats on the 9th day (after treatment with BSO as described in Fig. 1) revealed dramatic changes in the epithelial cells (Fig. 2*A*)—i.e., many vacuoles, apparent loss of cytoplasm, and mitochondrial swelling and degeneration. In contrast, there was much less change in the epithelium after treatment with both BSO and GSH monoester (Fig. 2*B*). A comparison of the epithelial zone of the lens of a control mouse 6 hr after birth with that from an age-matched mouse from a mother treated with BSO is shown in Fig. 3. Treatment with BSO led to substantial vacuole formation and mitochondrial swelling and degeneration. Examination of the nuclei (Figs. 2*A* and 4) indicated that BSO treatment led to increased density of the chromatin pattern, indentation, and shrinkage.

Transfer of BSO from Maternal Circulation to the Fetus. Administration of BSO is more effective in producing cataracts in rats and mice when given soon after birth (2–6 days) than when given later; no cataracts were found after treatment of animals older than 14 days. This may be related to changes in the blood supply of the lens during the period prior to opening of the eyes (28) and to the lower rates of metabolism in older animals (29, 30).

When pregnant mice were given BSO at different periods during gestation (i.e., from days 6–10, 9–13, or 15–19), cataracts were found in the offspring only after BSO was given on days 15–19. This period corresponds to that in which much of fetal eye development occurs (31). No cataracts were found in the BSO-treated mothers. Analyses done on the 18th day of gestation, after administration of BSO for 12 days, revealed the presence of substantial levels (0.14–0.32 μmol/g) of BSO in the fetal tissues and that the GSH levels of fetal and maternal tissues (liver, kidney, heart, lung, eye, placenta) were decreased to 16–38% of the corresponding control values. Examination of the fetal tissues by light and electron microscopy did not reveal abnormalities; lens epithelial cell damage (and cataracts) is the major consequence of GSH depletion under these conditions. However, more severe deficiency of GSH might well lead to other changes (cf. refs. 32–35).

DISCUSSION

Studies on BSO-induced cataract formation are consistent with previous observations on cataractogenesis. Spector and co-workers (36–39) found that oxidation of lens protein is an early event in cataract formation; that cataract formation is associated with formation of methionine sulfoxide, mixed disulfides, and cysteic acid in lens proteins; and that humans with cataracts have high levels of hydrogen peroxide in the aqueous humor. Several findings suggest that reactive oxygen species play a role in the development of lens opacities and lead to conformational changes and protein crosslinking

Table 1. Prevention of BSO-induced cataracts by GSH monoester

Exp.	Treatment	No. of cataracts/ no. of rats	Total lens GSH,* μmol/g	Mitochondrial GSH,* nmol per mg of protein
1	Controls	0/31	9.50 ± 0.50 (100)	11.0 ± 0.7 (100)
2	BSO	20/20, 11/11,† 11/11†	0.30 ± 0.03 (3)	2.3 ± 0.3 (21)
3	BSO + GSH	11/11	0.33 ± 0.04 (3)	2.4 ± 0.3 (22)
4	BSO + GSH monoester	0/20	0.70 ± 0.05 (7)	5.1 ± 0.3 (46)

Newborn rats were injected intraperitoneally with two daily doses of saline (n = 20) or Na_2SO_4 (2.5 μmol/kg) (n = 11) (controls; Exp. 1), or with BSO (Exps. 2–4) as described in Fig. 1. The rats were also injected twice daily with GSH (Exp. 3) or with GSH monoisopropyl ester (Exp. 4) as described in Fig. 1. The number of rats with cataracts (in all cases, bilateral) is given.

*Values are given as means ± SD (n = 3). Values in parentheses are percent control value.

†Also treated with two daily doses of Na_2SO_4 (2.5 mmol/kg) and with isopropanol or ethanol (5 mmol/kg).

in the lens (40–43). The core portion of the lens is devoid of cell organelles; the cells of the peripheral zone, which are rich in mitochondria, are of major metabolic and protective importance. These cells are equipped with enzymes that destroy peroxide and related oxidants, such as GSH peroxidases, superoxide dismutase, and catalase.

Mitochondria generate large amounts of hydrogen peroxide (44), and GSH seems to be required for its destruction. In GSH deficiency, peroxide (formed in mitochondria and at other sites) may enter the lens core and produce damage, leading to cataracts; high peroxide levels would also be expected to produce mitochondrial damage. Loss of mitochondria probably accounts for the significant (25–40%) decrease in lens weight gain after giving BSO (Fig. 1 legend). Mitochondrial loss, which would decrease the available cellular energy, probably also occurs in other tissues after BSO administration (13, 14) and affects absorptive processes, metabolism, and synthesis of macromolecules.

Mitochondria acquire GSH by transport from the cytoplasm and are able to retain some GSH in the presence of cytoplasmic GSH depletion (45). There seems to be a relationship between the extent of mitochondrial GSH depletion and cellular damage. Thus, muscle degeneration was found when the mitochondrial GSH was ≈20% of the control (13). Lamellar body and mitochondrial damage in lung type 2 cells occurred with mitochondrial GSH values that were 21% of the control (14), but no cellular damage was observed after prolonged treatment with BSO in liver and heart, which had mitochondrial GSH levels that were >40% those of controls. It is notable that administration of GSH monoester increases mitochondrial GSH levels in lens and in other tissues. Extramitochondrial effects of GSH deficiency probably also

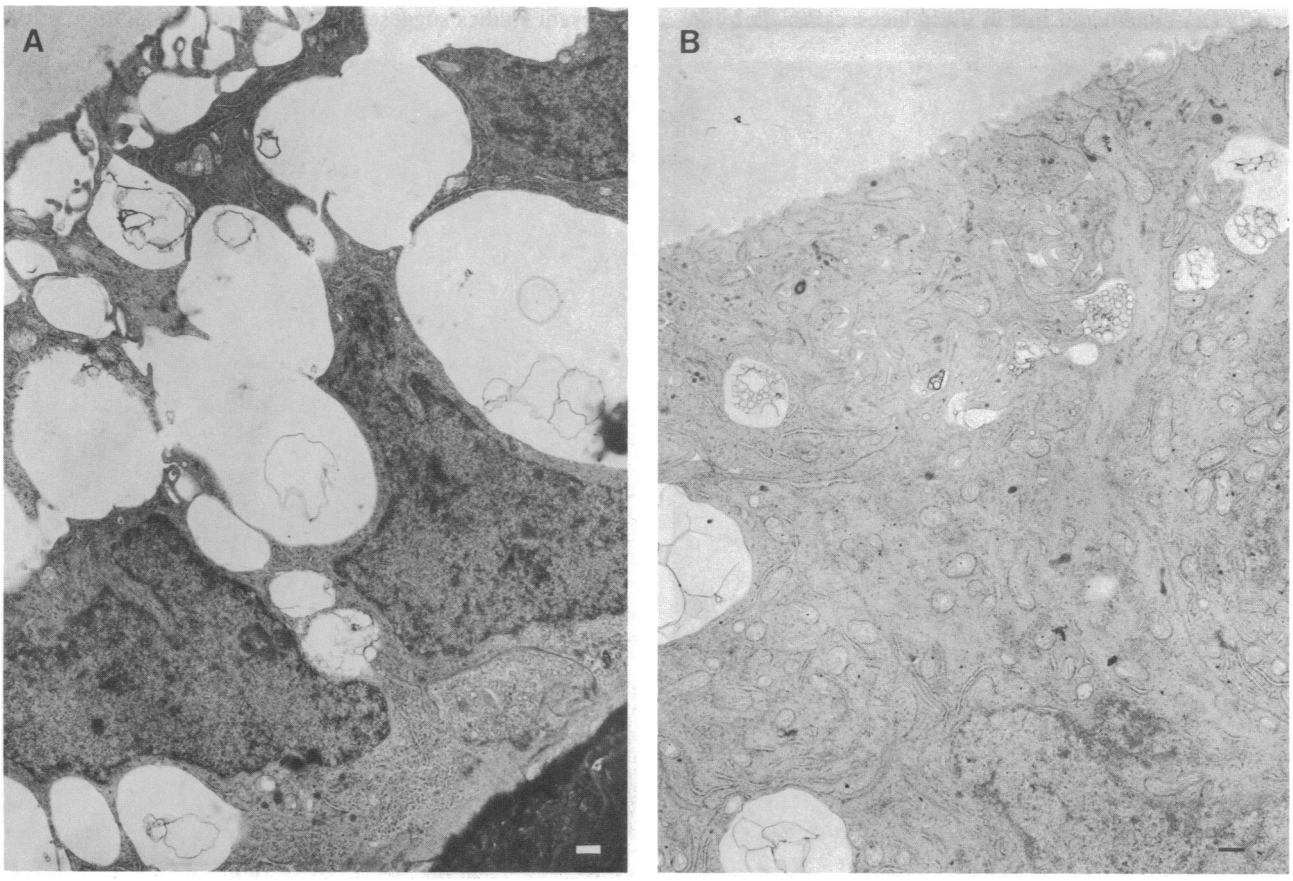

FIG. 2. Representative electron micrographs of the epithelial zone of the lens of a rat treated with BSO (*A*) and of a rat given BSO plus GSH monoisopropyl ester (*B*); 9-day-old rats were used after treatment described in Fig. 1. In *A*, there is marked loss of epithelia and mitochondria with vacuolization, mitochondrial damage, and nuclear changes. In *B*, a few vacuoles are present; otherwise, this resembles normal lens epithelium. The edge of the lens is shown in the upper left corner in both *A* and *B*. (Bar = 0.25 μm, ×8950.)

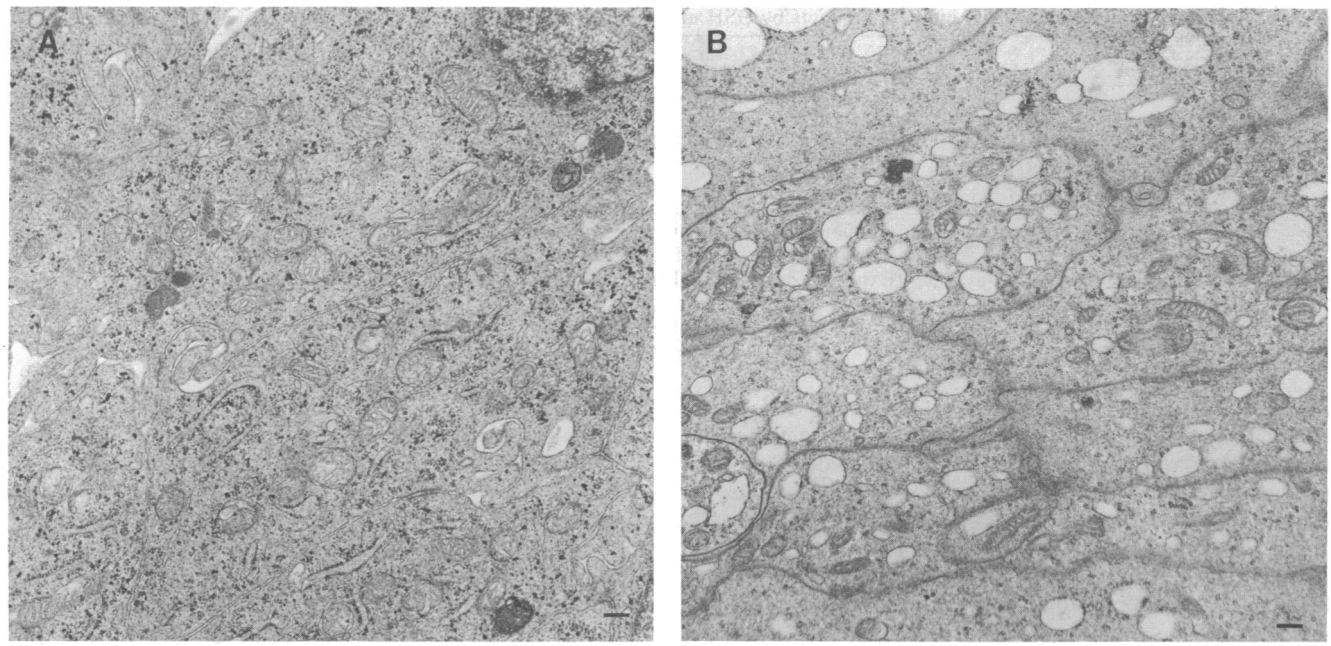

FIG. 3. Representative electron micrographs of the epithelial zone of the lens of a control mouse 6 hr after birth (*A*), and an age-matched mouse from a mother treated with buthionine sulfoximine (see text) (*B*). In *B*, there is marked mitochondrial swelling and degeneration and vacuole formation. (*A*, bar = 0.47 μm, ×17,150; *B*, bar = 37 μm, ×13,500.)

contribute to cataract formation; thus, electron microscopy showed changes in cell nuclei.

The finding that administration of GSH ester prevents development of cataracts in BSO-treated animals strongly supports the conclusion that BSO induces cataracts by inhibiting GSH synthesis in lens epithelial cells. Although it seems unlikely, it is conceivable that BSO exerts a toxic effect on cells separate from and in addition to its effect in inhibition of γ-glutamylcysteine synthetase. However, the present studies suggest that such hypothetical toxic effect of

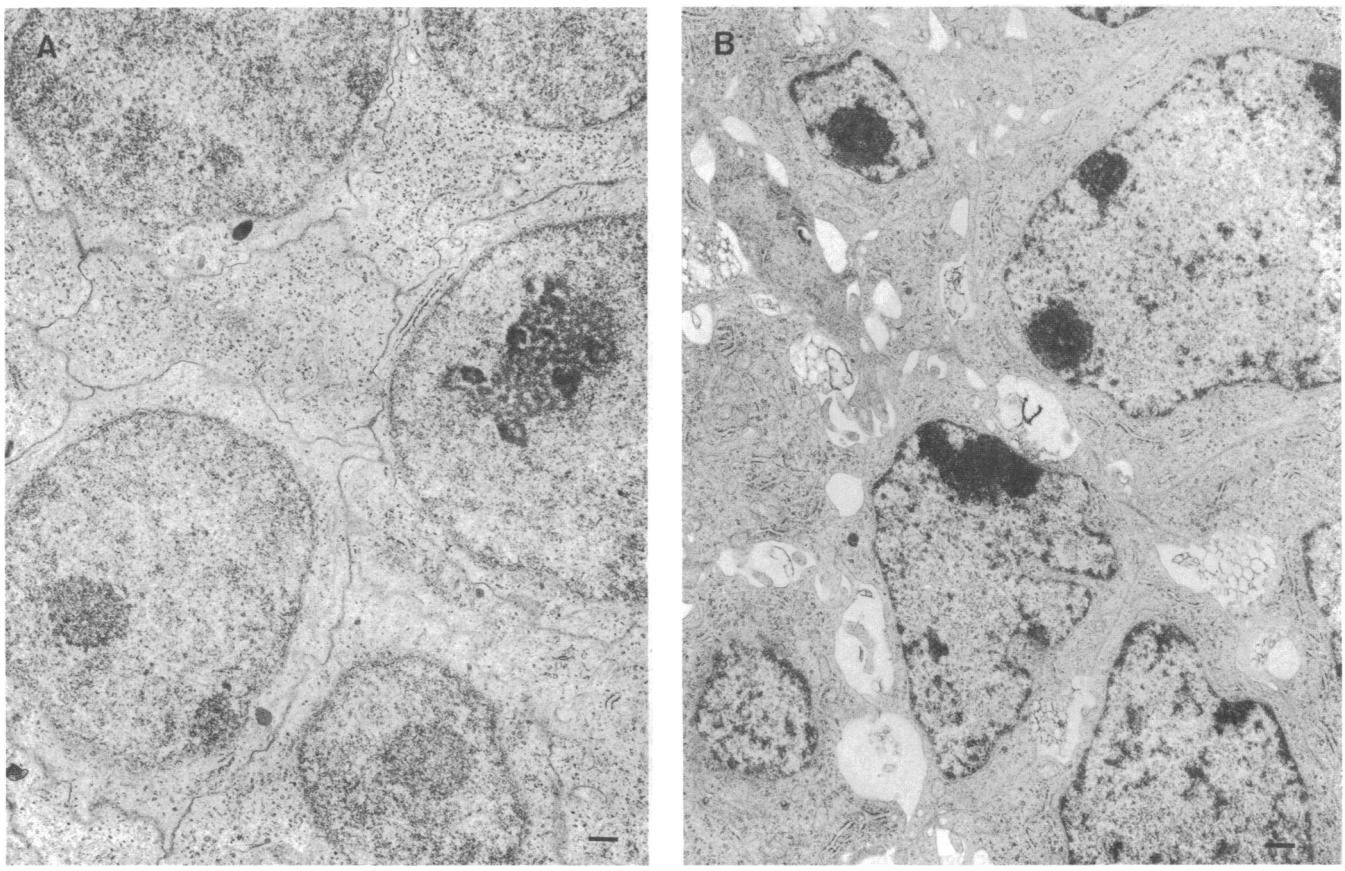

FIG. 4. Electron micrographs showing the nuclei of epithelial cells of a control (*A*) and a treated (*B*) mouse, as in Fig. 3. In *B*, there is an increase in the density of the chromatin pattern and nuclear indentation and shrinkage. (Bar = 0.21 μm, ×8800.)

BSO would be prevented by GSH. Reports describing "toxicity" of BSO have appeared (see, for example, ref. 46), and it was observed earlier that human lymphoid cell lines exhibited decreased viability after marked GSH depletion produced by treatment with BSO (47). Such effects most probably result from GSH deficiency. However, BSO can inhibit transport of γ-glutamyl amino acids (apparently competitively) (48). The physiological effects of molecules containing the sulfoximine moiety, apart from methionine sulfoximine and higher homologs of this compound that inhibit GSH synthesis (49), have not been extensively examined. Two higher homologs of BSO (including a D-isomer) were found to inhibit GSH synthesis and to exhibit lethal toxicity in mice (20). Administration of buthionine sulfone did not produce GSH deficiency in mice or structural damage to skeletal muscle (13) and lung (14); it did not produce cataracts in the offspring when given to pregnant mice.

That older rats and mice do not develop cataracts after administration of BSO may be ascribed, at least in part, to the development of an effective blood–aqueous humor barrier. Preliminary studies indicate that BSO is less readily transported into the eye in older mice than in mice <14 days old. It remains to be determined whether GSH esters are significantly transported into the adult eye; these and other derivatives of GSH need to be investigated with respect to their transport properties. The present and previous findings, which support the view that cataracts are produced by oxidation of lens proteins, suggest that procedures that increase lens GSH levels would protect against development of cataracts.

Turnover of GSH in lens is relatively slow; about half of the GSH present disappears ≈48 hr after giving BSO (Fig. 1) (cf. ref. 50). This compares with $t_{1/2}$ values (after giving BSO) of 60 min, 25 min, 20 min, 45 min, 5.5 hr, and 16 hr in liver, lymphocytes, kidney, lung, heart, and skeletal muscle (12–14).

We observed no deaths after administration of BSO to rats on the 2nd day of life or later; its administration on the 1st day of life was accompanied by ≈50% mortality for reasons that remain to be learned. About 10% of the rats given repeated doses of GSH monoester died between days 3 and 8; such mortality may probably be ascribed to the effects of the alcohol released. Newborn rats given equivalent amounts of either isopropanol or ethanol showed a 20% mortality. Neither GSH monoester nor alcohol is toxic to older rats at these doses.

The approach used here in which BSO is applied to inhibit GSH synthesis, and in which GSH monoester is used to restore cellular GSH, seems to be a good model for examination of the cellular functions of GSH. The developing lens, which is more sensitive to the effects of GSH depletion than the other tissues thus far examined, may be valuable in experimental work on the functions of GSH.

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