with [^{14}C]thymidine, an increased mitogenic effect was observed in the adipose cell clusters, demonstrating that the IGF-1 acted on cells that had already responded to growth hormone. As the effect was less marked than that obtained when the growth hormone and IGF-1 were present together for several days, these experiments do not rule out the possibility of an additional synergistic effect of IGF-1. However, because the cellular response to the hormone is not highly synchronized, the more pronounced effect observed when both agents are present is probably due to a greater likelihood that the IGF-1 will encounter the young adipose cells when they are most sensitive to it.

It is clear from these experiments that IGF-1 is a second effector in growth hormone action. Alone, it has a small mitogenic effect on preadipose cells, but it produces no differentiation. Together with growth hormone, it has a large mitogenic effect, but mainly on the differentiating cells. This results in clonal expansion of those cells, thereby selectively increasing their abundance. This process is completed within a few days, after which the adipose cells become mature enough so that they no longer multiply.

It has been demonstrated (*12, 13*) that insulin at supraphysiological concentrations has a mitogenic effect on cultures of Ob17 adipose cells. We found that all of the effects we describe for IGF-1 could also be produced by insulin at similar concentrations. Receptors for both IGF-1 and insulin increase with the differentiation of 3T3-L1 fibroblasts to young adipocytes (*14–16*). Although, in many cell types, mitogenic effects of both insulin and IGF are mediated by IGF receptors (*17*), in some cell types insulin receptors may also mediate mitogenic effects (*17–19*). However, since the serum concentration of IGF-1 is about 200 ng/ml, whereas that of insulin is usually about 1 ng/ml, it seems likely that clonal expansion in adipose tissue of the animal would be mainly the result of the action of IGF-1.

The dual effector theory of growth hormone action likely applies to other cell types. A direct effect of growth hormone on cartilage has been demonstrated in animals and in culture (*20–22*), although not yet clearly on prechondrocytes, as should be the case if the analogy to the adipose conversion is valid.

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Near-Total Glutathione Depletion and Age-Specific Cataracts Induced by Buthionine Sulfoximine in Mice

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The specific inhibitor of glutathione biosynthesis, L-buthionine sulfoximine (L-BSO), although relatively nontoxic in adult mice, induces severe glutathione depletion and age-specific pathological changes when repeatedly administered to male suckling mice. Dense cataracts developed when mice aged 9 to 12 days were given a series of injections of L-BSO, despite excellent survival and the absence of other significant long-term effects. By contrast, similar treatment of mice aged 14 to 17 days, although slightly less effective in reducing glutathione levels, resulted frequently in death, hind-leg paralysis, or impaired spermatogenesis, but did not produce cataracts. Administration of L-BSO to preweanling mice provides a novel model system for the induction of cataracts by depletion of lens glutathione and may enable the study of critical functions of glutathione in the lens and other growing tissues during early postnatal development.

GLUTATHIONE (GSH), γ-GLUTAMYLcysteinylglycine, the most abundant nonprotein thiol in living cells [0.5 to 10 m*M* (*1*)], is recognized to be a major intracellular antioxidant, a key component in the metabolism of cysteine and cysteine-containing proteins, and a broadly specific deactivator of potentially toxic electrophilic agents, via enzyme-catalyzed S-conjugation (*2*).

A number of compounds have been developed to alter the metabolism and intracellular concentration of GSH (*2*). One of the most widely used, in recent years, is buthionine sulfoximine (BSO) [(S-(*n*-butyl) homocysteine sulfoximine], a specific inhibitor of γ-glutamylcysteine synthetase (*3*), the enzyme that catalyzes the first step of GSH biosynthesis. Over 90 percent reduction of GSH levels has been achieved in cells cultured in the presence of D,L-BSO (*4*), a mixture of four stereoisomers, or the more specific preparation, L-BSO (L-buthionine-*S*,*R*-sulfoximine) (*5*). The consequences of such in vitro exposure include increased sensitivity to radiation, chemotherapeutic drugs, thermal stress, and oxidative agents (*4, 6*), inhibition of the biosynthesis of leukotriene C and prostaglandin E_2 in macrophages (*7*), and teratogenesis (*8*). On the other hand, treatment of adult mice with BSO has resulted in significant retention of GSH (at least 20 percent of control values) in most tissues and no grossly observable pathological effects (*9*). This and the large doses of BSO required for a significant effect on GSH levels in vivo have limited the usefulness of this highly specific drug for general physiological surveys of GSH function.

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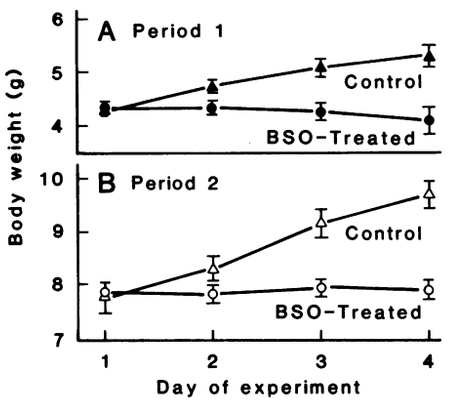

Fig. 1. Daily weight determinations in control and BSO-treated mice. (A) Period 1 (9 to 12 days of age). (B) Period 2 (14 to 17 days of age). Each point represents the mean ± SEM of four saline-injected control or four experimental animals raised in the same cage with a single nursing female.

During studies originally designed to evaluate the effects of GSH depletion on spermatogenesis, preweanling mice, in contrast to adult mice, were found to be highly sensitive to repeated administration of BSO. Multiple injections of L-BSO (4 mmol per kilogram of body weight), as described below, caused severe reductions of GSH concentration in four different tissues and had dramatic effects on the appearance and well-being of the animals.

Male Swiss-Webster mice, derived from pregnant dams (CAMM), were raised from birth (0 days of age) and were treated for a 3-day period, either at 9 to 12 days of age (period 1) or at 14 to 17 days of age (period 2). The animals were lightly anesthetized with ether and given subcutaneous injections four times daily, at 2- to 2.5-hour intervals. Experimental mice received 0.20*M* L-BSO plus 0.10*M* NaCl at 20 μl per gram of body weight. Controls received an equivalent dose of 0.30*M* NaCl. Unless otherwise noted (see legend to Fig. 1), controls and drug-treated mice were housed separately. The body weight of each animal was determined daily. Final injections were administered respectively at 10 a.m. on the day the animal was killed for assay of GSH (extended protocols, 1-e and 2-e) or at 6 p.m. on the previous day (short protocols, 1-s and 2-s). The mice were killed at 2 p.m. (1-e and 2-e) or at 10 a.m. (1-s and 2-s). The time between final injections and removal of organs for GSH determination was thus about 4 hours in the extended protocol and 18 hours in the short protocol. Total glutathione concentrations in several tissues were compared by means of an enzymatic cycling assay that does not distinguish between GSH and glutathione disulfide (GSSG) (*10*). The resulting values, expressed in GSH equivalents, were assumed to closely approximate the actual concentrations of GSH in the excised tissues (see legend to Table 1).

Under all conditions studied, GSH concentration markedly decreased in liver, kidney, testis, and lens (Table 1). The particularly severe reductions during period 1 reflect nearly complete depletion of the major fraction of GSH in the cytosol and imply substantial reduction of the more slowly metabolized pool of mitochondrial GSH (*11*). GSH concentrations 1 to 4 percent of control values were seen in liver, kidney, and testis removed 4 hours after a final injection on experimental day 4 (period 1-e). The concentrations of GSH were somewhat higher (4 to 16 percent of control values) in the same tissues of animals allowed to recover overnight after the last injection on experimental day 3 (period 1-s).

In all tissues examined, BSO was less effective in reducing GSH levels in period 2 than in period 1. This contrast may reflect developmental changes in the metabolism of either GSH or BSO in the preweanling mice. Especially noteworthy is the value of 1.29 μmol/g for liver GSH in mice treated during period 2-s, as opposed to 0.21 μmol/g after treatment during period 1-s.

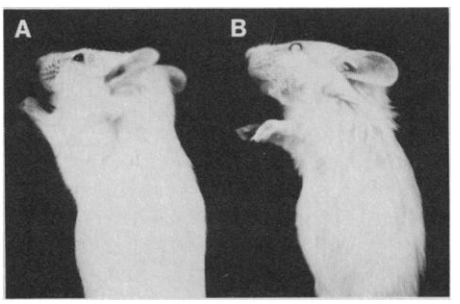

Fig. 2. Two male littermates at 18 days of age, after treatment during period 1-e. (A) Saline-injected. (B) BSO-injected. Mouse B, recovering from temporary diffuse alopecia, has dense cataracts and ruffled fur.

The most striking depletion of GSH occurred in the lens during period 1. This correlated with the development of opacities. Of 16 lenses from mice killed 18 hours after injection (period 1-s), only one remained transparent. The pool of four lenses that included this clear lens yielded a mean concentration of 0.034 μmol per gram of tissue. Three other groups of lenses, all opaque, were assayed similarly. These contained no measurable GSH, suggesting that a minimum level of GSH was necessary to maintain lens clarity at the time of the assay (around 12 days of age).

Generalized effects of L-BSO were evident in all animals treated with the drug for 3 days during period 1 or period 2, and were typified by lethargy, emaciation (Fig. 1), and fur abnormalities (Fig. 2). These symptoms began to disappear within 2 days after the cessation of treatment during period 1,

Table 1. Determinations of GSH concentration in tissues of mice injected with L-BSO or saline (see text). Tissues were homogenized in 15 to 100 volumes of 0.1*M* HCl and analyzed for GSH content after deproteinization with $HClO_4$ (*20*). The enzymic assay used (*10*) yields a measure of total glutathione [both GSH and its disulfide (GSSG)]. However, the latter is found in relatively minute concentrations in mammalian cells (*23*). Therefore, the concentration of GSH + GSSG in each of the above determinations is assumed to be essentially equivalent to that of GSH in the living tissue. Data are expressed in micromoles of GSH equivalents per gram and represent the means of four to seven determinations ± SD. For each tissue, significance of the differences between means was determined by rescaling (+0.1 units) and logarithmic transformation of individual values to equalize sample variance (*24*), followed by three-way analysis of variance (liver, kidney, testis) or two-way analysis of variance (lens), and Duncan's new multiple range test (*25*). Within each set of tissue values, those means with no common superscripts are significantly different at $P < 0.05$.

Treatment	Hours after treatment	Tissue GSH levels (μmol/g)			
		Liver	Kidney	Testis	Lens
Control, period 1-e	4	5.25 ± 0.70[a,d]	1.50 ± 0.17[a]	3.01 ± 0.25[a]	
L-BSO, period 1-e	4	0.06 ± 0.02[b]	0.06 ± 0.02[b]	0.05 ± 0.01[b]	
Control, period 1-s	18	4.65 ± 0.99[a]	1.46 ± 0.08[a]	3.34 ± 0.35[a]	3.97 ± 0.22[a]
L-BSO, period 1-s	18	0.21 ± 0.04[c]	0.24 ± 0.02[c]	0.31 ± 0.09[c]	<0.01[b]
Control, period 2-e	4	5.28 ± 0.38[a,d]	2.19 ± 0.19[d]	4.08 ± 0.12[d]	
L-BSO, period 2-e	4	0.20 ± 0.05[c]	0.22 ± 0.04[c]	0.48 ± 0.05[e]	
Control, period 2-s	18	6.96 ± 0.99[d]	2.18 ± 0.22[d]	4.12 ± 0.19[d]	4.26 ± 0.36[a]
L-BSO, period 2-s	18	1.29 ± 0.27[e]	0.38 ± 0.09[e]	0.54 ± 0.11[e]	0.24 ± 0.04[c]

resulting in 100 percent survival for at least 10 days in 14 animals not killed immediately for GSH assays. By contrast, 30 percent of mice (9 of 28) that were tested similarly after treatment during period 2 died 1 to 4 days after BSO administration was discontinued despite the somewhat milder depression of GSH levels during period 2 (Table 1).

Cataractogenesis, although restricted to mice treated during period 1, was, by far, the most well-defined and consistent anomaly observed. Opacification was obvious when the eyes first opened at age 14 to 15 days and progressed until the cataracts became total (pearly white) or near-total by approximately 18 days of age (Fig. 2). Slit-lamp biomicroscopy of 14-day-old mice revealed a relatively dense opacity in the anterior cortex and a particularly dense opacity in the nuclear region of the lens. These could be visualized through a typically less involved and more transparent central superficial cortex. At 21 days, the entire lens had become densely opaque (Fig. 3A). Thereafter, as lens growth produced new fibers, an essentially uninvolved outer cortex developed. By contrast, the lenses of mice treated with BSO in period 2 did not develop cataracts (Fig. 3B).

Although earlier studies have shown a correlation between cataractogenesis and reduced GSH levels (*12*), this is, to our knowledge, the first instance in which cataracts have been induced by a drug specifically designed to deplete cellular GSH. The BSO-induced cataracts may share a mechanism common to nuclear cataractogenesis arising from selenite administration to suckling rats (*13, 14*). However, such selenite-induced cataracts are not caused simply by depleting lens GSH, since GSH concentrations remain within 50 percent of control levels after a cataractogenic dose of selenite (*14*). By comparison, a single injection of BSO at 10 days of age in the rat, which lowers lens GSH to 4 percent of control levels within 4 days, does not produce opacities (*14*).

The consequences of artificially induced or congenital GSH deficiency in adult mammals already reported by others include necrosis of the liver (*15*) and gastric mucosa (*16*), hemolytic anemia (*17, 18*), and neuromuscular defects (*18*). We have, in fact, observed that paralysis of the hind legs can develop at approximately 19 to 20 days of age, after injection of BSO during period 2 (detected in 6 of 28 mice). This effect appears to be age-specific, since it has been observed only after treatment during period 2.

Negative long-term effects of GSH depletion on spermatogenesis were likewise confined to period 2. These were assessed at 50 days of age by examination of mature sperm samples obtained from the cauda of the epididymis, by the use of techniques described previously (*19*). Oligospermia (0.1×10^6 to 1.2×10^6 sperm per epididymis) was observed in four of five animals, after exposure during period 2-e. Motility in the oligospermic samples was either absent or subnormal (0 to 50 percent steadily beating tails). By contrast, all four animals evaluated similarly after treatment during period 1-e had ample stores of viable sperm (6.2×10^6 to 12.6×10^6 sperm per epididymis; 70 to 90 percent motility). The potential importance of GSH in spermatogenesis is discussed elsewhere (*20*). It is not clear, however, whether the differential effect of GSH depletion during period 2 was direct or was the consequence of the poor health of the treated mice.

Despite a report indicating that BSO may influence biochemical processes other than GSH biosynthesis (*21*), it is likely that the major pharmacological effects of BSO stem from the specific inhibition of γ-glutamylcysteine synthetase and consequent depletion of GSH (*22*). Therefore, its administration to preweanling mice may prove useful as a model for the early stages of γ-glutamylcysteine synthetase deficiency, a syndrome in humans characterized by hemolytic anemia and neuromuscular disorders (*18*).

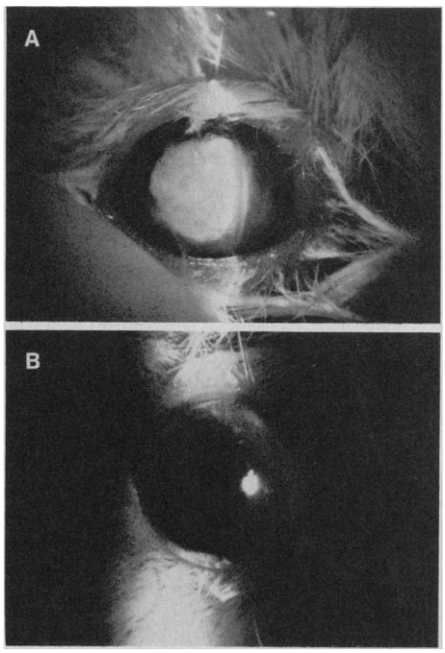

Fig. 3. Slit-lamp biomicrographs of eyes from BSO-treated mice, 9 days after the last injection of the inhibitor during (A) period 1-e or (B) period 2-e. The dense cataract in (A) contrasts sharply with the total transparency of (B). Lenses of mice treated during period 2 were indistinguishable from those of controls.

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