THE ACTION OF SELENITE ON ATP SYNTHESIS IN RAT LENS

by

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(ABSTRACT)

A subcutaneous injection of sodium selenite (30 umol/kg body weight) in 10-day old rats produced a cataract within 72 hours. Lens opacification was preceded by a 15% decrease in ATP content. Lens ATP did not fully recover to control concentrations by 11 days post-injection. A moderate correlation existed between lens weight and total ATP content in control lenses (r=0.509, n=153), but this relationship disappeared with selenite treatment (r=0.023, n=153). There was a significant inhibition by selenite on lens growth from 4 through 11 days after an injection.

In vitro exposure of lenses to 1.0mM selenite for 4 hours caused lens ATP to decrease 15%. The interaction of selenite with typical inhibitors of energy metabolism, azide for mitochondria and Cu^{+2} for glycolysis, indicated selenite affected lens ATP by inhibiting mitochondrial function. In contrast to in vivo studies, lens lactate production decreased in the presence of selenite in vitro. The decrease in lactate production may be

attributed to an increase in membrane permeability caused by selenite.

Both *in vitro* and *in vivo* exposure of lens to selenite decreased total glutathione concentration 25%. Experiments with buthionine sulfoximine, a specific inhibitor of glutathione synthesis, revealed that glutathione concentration can be depleted to 90% of controls with neither an effect on lens ATP nor cataract formation. These results suggest a decrease in lens ATP may be of greater importance in cataractogenesis than a reduction in lens glutathione.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

Cataracts are responsible for compromising visual acuity in man, and are a major cause of blindness worldwide. In 1982, the World Health Organization estimated that cataracts accounted for 17 million cases of blindness out of 42 million blind people (1). Epidemiological studies indicate that there is a greater frequency of cataract in developing nations than in the United States or Europe (2). Some progress has been made in reducing blindness due to infection and nutritional deficiency in third world countries. In contrast, senile cataracts, congenital cataracts, radiation-induced cataracts, and cataracts resulting from trauma, toxic chemicals or metabolic lesions are not easily prevented. Presently, the only means to rectify this visual impairment is through surgery. In the United States, approximately 357,000 lens extraction operations were reported in 1978 (2).

In order to establish preventive treatment for cataracts, it is imperative to understand the changes in lens metabolism during cataractogenesis. Studies with human cataractous lenses are limited by sample availability, and variability; hence, various animal models have been introduced (3). The selenite-induced cataract presents a consistent, efficient model to study

cataract in a mammal. A subcutaneous injection of sodium selenite (20 umol/kg body weight), in 10-day old rats, produces cataract within three days in 100% of the animals injected (4). Thus, the selenite-induced cataract presents a reproducible means for investigating metabolic anomalies involved in the development of cataract in vivo.

Previous studies with the selenite-induced cataract indicated reduced ATP/AMP ratios, with a maximum depression in lens ATP concentration by 24 hours following an injection of selenite. The change in lens ATP was accompanied by elevated lactate production (5). Apparently, selenite disrupted lens energy metabolism in the process of cataractogenesis. How the perturbation of lens ATP concentration is related to cataract formation is still unclear.

There is a need to understand the means by which selenite reduces lens ATP. Early literature describes selenite as a mitochondrial uncoupler, while other reseachers found it acted as an inhibitor of glycolysis in yeast cells (6,7). The following research was directed toward establishing how lens ATP concentration responds to selenite treatment. Determining how selenite disturbs lens ATP synthesis during selenite-cataract will

be valuble in understanding how a perturbation in energy metabolism is related to the sequential events in cataract formation.

II. LITERATURE REVIEW

The mammalian lens, a transparent, resilient, biconvex tissue, focuses light upon the retina in the visual process. The lens remains distinct from other organs as it is avascular, devoid of neural tissue, synthesizes unique proteins, and it contains only a monolayer of epithelial cells from which all other cells are derived. The most distinguishing characteristic of the lens is its ability to conserve its cellular history. The process of development and maturation is reflected by the retention of concentric cell layers throughout the lens lifetime with the oldest cells in the central nucleus of the lens.

Development & Maturation

The lens is encased in an acellular, transparent capsule which is derived from the basal epithelial layer of the lens. The capsule, composed of collagen and mucopolysaccharide, acts as a protective layer and grows in size and thickness to accommodate the growing lens. The capsule is permeable to nutrients and oxygen which are supplied by the surrounding aqueous and vitreous humors. Zonular ligaments extend from the equator of the capsule surface to suspend the lens behind the cornea and iris.

A single layer of epithelial cells lies beneath the

anterior region of the capsule. The central epithelial cells do not actively divide, although mitosis may be stimulated in response to hormones (8). The pre-equatorial region contains mitotically active epithelial cells. As the terminal cells of this region divide, the daughter cells migrate to the equatorial region where they begin to elongate in the process of differentiating into fiber cells. This elongation is accompanied by a 90° rotation of the cell such that the apical end of the cell moves toward the anterior of the lens (8).

Cell elongation and the rotational shift result in a bow formation in the fiber cell region. As the cell continues to elongate, the basal end reaches the central area of the posterior capsule while the apical end extends toward the anterior epithelium. When new fiber cells are formed, the apical and basal ends of the existing fibers losen their attachment from the epithelium, and interdigitate with the newly formed fiber cells to form anterior and posterior suture planes (2). The fiber cells located in this intermediate region of the lens constitute the lens cortex.

With the generation of new fiber cells, the older cells are displaced and converge toward the center of the lens, thus forming concentric cell layers. During the

maturation of the fiber cells, the cells become enucleated, gradually lose cellular organelles, and become more irregular in shape. The central fiber cells comprise the lens nucleus.

Thus, various cell types within the lens are derived from a germinative region in an epithelial monolayer. Cell differentiation results in the formation of three distinct regions of the lens; the nucleus, the cortex, and the epithelial layer, which are encased by the lens capsule. Not only are these regions characterized by a progressively changing cellular morphology, but they reflect an age gradient in a tissue that retains all the cells it has ever generated.

Cellular morphology

Epithelial cells - Epithelial cells are cuboidal in shape, contain large nuclei and typical cellular organelles. Since the lens has no blood supply, there is a requirement for cell-to-cell communication and the passage of intercellular materials. The lateral and apical plasma membranes of epithelial cells have junction specializations to satisfy the needs of the lens. Desmosomes, which function in cell adhesion, are dense plaques located on the cytoplasmic side of plasma membranes of two opposing cells (9). Gap junctions, also occur along the membrane of the lens epithelial cells,

and contain channels which traverse the membrane and permit the passage of ions and metaboltes of 600-700 daltons (9).

Fiber cells - Hexagonal fiber cells comprise the cortical and nuclear regions of the lens. The fiber cells are contoured with surface modifications and interdigitations to maintain fiber cell opposition, and prevent cell slippage during lens accomodation. The fiber cells of the bow region are fashioned with finger-like projections which interlock with notches in adjacent cells, whereas internalized fiber cells possess ridges on the surface in addition to the interlocking processes. Fiber cells are further characterized by junction specializations. Although desmosomes are lost during maturation, fiber cells still possess gap junctions, which are often found associated with surface projections (9).

Structural changes accompanying aging - The lens is responsible for maintaining its native components throughout a lifetime, with the homeostasis of the nuclear region being sustained by the metabolically active periphery. Aging imposes increased stress on the lens. The increase in lens volume relative to the aqueous and vitreous humors limits the supply of oxygen and nutrients (10). Moreover, it is the mass of the fiber

cells that increases, while the metabolic activity of the epithelial cells is reduced (11). Therefore the tissue becomes more susceptible to irreversible damage.

Cytological alterations develop with aging in the lens epithelial and fiber cells. An overall reduction in size of the epithelial cell is accompanied by a decrease in the number of organelles, condensation of chromatin and mitochondrial swelling. Fiber cells of the bow region become irregular in size and shape, while surface discontinuities of cortical fiber cells result in an increased number of surface projections penetrating the lens capsule. Aging fiber cells also develop atypical mitochondria. These abberations in cell structure culminate in fiber cell swelling and disintegration at the sutures. Epithelial cell differentiation begins to slow down prior to the disintegration of lens fiber cells (8).

Aging in the lens is reflected by pronounced changes in its structural proteins, the crystallins. The lens must maintain its highly ordered protein environment in order to prevent light scattering, yet it is unable to replace damaged cells. Therefore, since the proteins of the nucleus represent the oldest proteins in the lens, they have the greatest chance of accumulating age-related modifications.

The lens fiber cells are composed of three protein groups, the α-, β-, and γ-crystallins. The change in relative distribution of the crystallins with age is reflected in the different lenticular layers. A primary observation in the aging process is the accelerated process of protein insolubilization (8,11,12,13,14). Bindels, et al. have reported a 40% increase in the water insoluble fraction of the aging rat lens (14). Using radiolabeling techniques, they have determined that the insoluble fraction is ultimately derived from certain water-soluble crystallins. The α-crystallins and β-L-crystallins which decrease during aging, are incorporated into water insoluble protein as they form high-molecular weight aggregates and β-H-crystallin, respectively. A 5-fold reduction of the γ-crystallin has been observed in the year-old rat lens. Bours and Hockwin suggest that the decrease in some of the γ-crystallins may also be attributed to insolubilization (14).

LENS METABOLISM

The ultimate purpose of lens metabolism is to maintain lens transparency. Low water content and a high concentration of ordered protein is required to maintain a uniform index of refraction. Failure of lens

metabolism to sustain a uniform index of refraction will result in the formation of a lens opacity.

Carbohydrate Metabolism

The lens receives its nutrient supply from the surrounding aqueous humor. The primary energy source for the lens is glucose, which is transported into the lens by facilitated diffusion. A 30% elevation in lens insulin concentration after feeding suggests insulin may function in sugar transport (9). The presence of high affinity receptors for insulin on lens epithelial cells is consistant with a role for insulin in glucose transport (15). Depletion of ATP and proteolysis occurred when lenses were incubated in glucose-free medium (9).

Glycolysis - Since there is limited oxygen available to the lens, lenticular energy is derived predominantly from anaerobic glycolysis (16). The glycolytic rate is regulated by hexokinase which is controlled by lens concentrations of glucose-6-phosphate and ATP. The activities of hexokinase, and phospofructokinase in the lens epithelium remain constant throughout life. However, reduced activities in the cortical regions of human and bovine lenses have been reported (3,9,17). The majority (70-90%) of the lactate produced by this process diffuses from the lens into the aqueous humor, while the

remainder may be oxidized by the citric acid cycle (18). Therefore, under anaerobic conditions, ATP production by glycolysis almost entirely satisfies the energy needs of the lens (19)

Sorbitol Pathway - The sorbitol pathway produces sugar alcohols and ketose sugars via aldose reductase and polyol dehydrogenase, respectively. The $K_m$glucose reported for hexokinase and aldose reductase in human lenses is 0.1mM, and 28mM, respectively (16). In the presence of high concentrations of glucose, hexokinase becomes saturated, and the glucose may enter the sorbitol pathway. The products of this pathway are neither membrane permeable nor rapidly metabolized (15). Consequently, increased sugar alcohol content elevates the osmotic pressure, causing swelling and rupture of the fiber cell, and may culminate in cataract formation. The accumulation of polyol depends the activity of aldose reductase and polyol dehydrogenase relative to hexokinase, and the rate of product diffusing through the cell membrane (16). Elevated levels of sorbitol in lenses from diabetic adults may result from reduced hexokinase activity (16). Therefore, a high aldose reductase/hexokinase ratio, concomitant with elevated glucose predisposes the lens to cataract formation (19).

The biological function of the sorbitol pathway is uncertain. Dihydroxyacetonephosphate may be formed from glucose through the sorbitol pathway when hexokinase is rate limiting (9). This glycolytic bypass may be of particular importance in the aging lens. While it is known that certain glycolytic enzymes become less active with aging, the activities of aldose reductase, polyol dehydrogenase, and ketohexokinase remain constant or increase during aging (20). Secondly, the pathway may act as an osmotic buffer. Fluctuation in extracellular glucose levels may compromise lens permeabilty due to osmotic shock. Intracellular sorbitol production may offset lens dehydration due to rapid elevations in glucose (9).

Aerobic metabolism - aerobic metabolism accounts for 30% of the ATP produced in the bovine lens and 33% in rat lens (21, 22). The TCA cycle plays a minor role in the lens energy supply, largely because cells containing mitochondria are found only in the epithelial monolayer. Trayhurn and van Heyningen demonstrated that bovine lenses incubated without glucose retained their metabolic integrity under aerobic conditions. They suggest that the lens utilizes endogenous substrates when glucose is not present (23). Glutamate or glutamine has been

suggested as a likely candidate for an endogenous substrate.

Glutathione Metabolism

Glutathione occurs predominantly in the lens epithelium where it is synthesized. Lens epithelium synthesizes its glutathione. Synthesis of GSH may utilize 11% of the ATP produced from glycolysis (9). Although the concentration of glutathione has been reported to diminish with aging and cataract formation, the precise role of glutathione in lens metabolism is unclear.

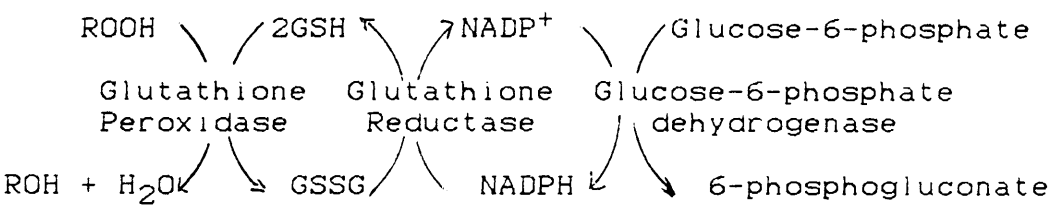
Glutathione is catabolized in the lens via the γ-glutamyl cycle (24). The enzyme γ-glutamyl transpeptidase transfers γ-glutamyl group from GSH to a receptor amino acid. The amino acid conjugate is converted to free amino acid and 6-oxoproline by γ-glutamylcyclotransferase. Free glutamic acid is subsequently generated from oxoproline by oxoprolinase (24).

Glutathione is believed to maintain the highly ordered protein structure by reducing disulfide bonds of lens crystallins. Spector has found elevated disulfide content in high-molecular weight proteins from human cataract (25). The random formation of disulfide bonds

may initiate protein aggregation and opacification of the lens.

Indirectly, glutathione preserves lens transparency by protecting the Na^+/K^+ ATPase from thiol oxidation. The Na^+/K^+ ATPase maintains Na^+/K^+ ratio. Reddy and Giblin have reported that exposing lenses to 1mM t-BHP decreased the concentration of glutathione 25%, and irreversibly impaired the activity of the Na^+/K^+ ATPase compared with controls (24). Apparently, sulfhydryls involved in cation transport become susceptible to oxidation when glutathione levels are reduced.

Glutathione is involved in the enzymatic detoxification of H_2O_2 in the lens. The interaction of glutathione with the glutathione peroxidase-glutathione reductase system and the hexose-monophosphate shunt protect the lens from peroxidative damage.



The relationship between carbohydrate and glutathione metabolism is demonstrated by an increased rate of GSSG formation, and stimulated production of NADPH by the hexose-monophosphate shunt upon exposure to t-BHP or

H_2O_2 (26). Additional studies by Giblin and McCready indicate that lenses with impaired glutathione reductase activity are more susceptible to oxidative damage by H_2O_2 (26).

Others have reported reductions in lens glutathione in cataractous lenses (3). The relative importance of glutathione in maintaining lens clarity has been demonstrated by Calvin, et al (36). Repeated subcutaneous injections of buthionine-sulfoximine, a specific inhibitor of gama-glutamylcysteine synthetase, decreased glutathione content of the liver, kidney, testis, and lens in 9-12 day old mice. Dense nuclear and cortical cataracts were visible as soon as the eyes were opened; no glutathione was detected in the cataractous lenses. These data suggest a minimum level of glutathione is necessary to maintain lens clarity.

CATARACT

A cataract has been described as an opacity of the lens sufficient to impair vision (22). Opacities develop when a uniform refractive index is no longer maintained. A change in refractive index causes light scattering, and impairs visual function. A mature cataract represents the final manifestation of a degenerative process in the lens.

The loss of lens transparency, leading to cataract formation may be initiated by insults including radiation, toxic chemicals, congenital abnormalities, metabolic lesions, nutritional deficiencies, trauma, aging, and enocrinological abnormalities such as hypocalcemia. (3). Although there are distinctions in the pathogenesis of cataract induced by these specific insults, common features of cataract are also evident.

Protein modifications

During cataractogenesis, the lens proteins become more insoluble. With aging, normal lenses also accumulate high molecular weight aggregates (HMW), and insoluble protein. However, there is a distinction between the insoluble protein and HMW found in the normal aging lens and the cataractous lens. In normal lenses, insoluble protein is predominantly found in the nucleus, and contains weak, non-covalent bonds. Conversely, the insoluble protein, and HMW from cataractous lenses are not restricted to the nucleus, and the polypeptides contain covalent linkages (11). The covalent linkages appear in two forms, disulfide and nondisulfide bonded (27).

SDS agarose-polyacrylamide electrophoresis of the insoluble protein fraction from cataractous lenses reveals a distinct HMW which does not penetrate the gel,

but disappears when reduced with B-mercaptoethanol. The proteins of normal lenses do not show any change upon reduction (11, 27). These data are consistant with disulfide linkages in the cataractous insoluble protein fraction.

Oxidation

Proteins isolated from cataractous lenses show increased oxidation of water soluble and insoluble proteins with increasing severity of the cataract. Events initiating oxidative reactions, with consequent formation of disulfide aggregates, involve alteration of membrane structure. Garner and Spector have shown that although sulfur oxidation occurred in all fractions of the cataractous lens, the most extensive oxidation appeared in the 43,000 dalton extrinsic membrane protein fraction (28). Virtually all the methionine and cysteine in the water-insoluble protein fraction was oxidized to methionine sulfoxide and cysteine disulfide, respectively.

Membrane transport systems responsible for maintaining osmotic balance within the lens are also targets for oxidative damage. Altered intracellular concentrations of Na^+ and K^+, diminished Na^+/K^+ ATPase activity, and an accumulation of Ca^+ were observed during lens opacification (29). H_2O_2 inhibited the influx of

^{86}Rb in cultured bovine lenses by modifying the Na^+/K^+ ATPase (30). Alternately, Lucas et al. indicated that human cataractous lenses with high sodium and calcium accumulation had an increase in membrane permeability (31). The concept that altered electrolyte status in human senile cataracts may be attributed to a defect in membane permeability is further supported by the work of Mariani and Pasino (32).

It is likely that the oxidant for these reactions is localized in the aqueous humor. Spector has reported elevated H_2O_2 concentration in the aqueous humor of lenses from patients with cataract (30). Moreover, the lens contains high concentrations of ascorbic acid which may generate H_2O_2 in the presence of light (27). An alternative oxidizing agent that may be involved in initiating cataract is oxygen itself. Elevated oxygen tension in ocular humors may accelerate the formation of active oxygen species, which can disrupt lipid membranes and cause enzyme inactivation (33,34,35). Antioxidant enzymes, catalase, superoxide dismutase, and glutathione peroxidase, may be less active in cataract. In addition to the oxidation of cysteine and methione, a concurrent loss of glutamic acid and glycine from cataractous lenses suggests the presence of glutathione-protein mixed disulfide (28).

SELENITE-INDUCED CATARACT

Selenite Cataracts

The transparency of the crystallin lens is affected by selenium status. Severe selenium deficiency produced cataract in second generation rats, due to decreased efficiency of glutathione peroxidase (37). Conversely, Ostadalova, Babicky and Obenberger induced the formation of bilateral nuclear cataract with a single dose of sodium selenite (20 umol/kg body weight) (4). This observation has been confirmed by others (38,37). Shearer, Anderson, and Britton have shown that oral administration of selenite also resulted in cataract formation (39). The effect of selenite correlated with the age of the animal. The neonate rat was more resistant to the lethal action of large doses of selenite, but was more susceptible to cataract formation (40).

The influence of trace minerals and other seleno-compounds on cataractogenesis has been studied. Sodium selenate, and the selenoamino acids, selenomethionine, and selenocystine, induced cataract, although no opacity was apparent following an injection of methylated seleno compounds (40). In addition to selenite, Shearer, et al. investigated the effect of fourteen other trace minerals on cataractogenesis (39). No other trace minerals

induced cataract formation, yet trace mineral interaction with selenite influenced the frequency of cataract. Mercury prevented selenite-induced cataract. Mercury can complex with selenite to diminish the toxicity, or it may prevent selenite from binding to sulfhydryls by competing for binding sites (39).

Histological changes - The selenite-induced opacities appear as a bilateral, nuclear cataract (3,41). Shearer, et al. described the different stages in the development of the selenite cataract (41). During the first 24h, well defined peripheral posterior opacities appear, accompanied by swollen fiber cells, small vacuoles and pre-equatorial haziness. The post-equatorial cataract disappears within the next 48 hours, and the nucleus becomes enclosed in a layer of swollen fibers. A central, defined, bilateral, nuclear cataract and pronounced swelling of the perinuclear fibers appear in the third stage. The nuclear cataract becomes more dense and more angular 5-10 days following the selenite injection.

The uptake of ^{75}Se was localized to the cortical region of the lens in association with lens proteins. Recent studies focusing on the cortical cataract resulting from selenite treatment, indicate rapid damage to the lens epithelium (42). Supressed mitotic activity

was evident in the germinative zone 5 hours following an injection of selenite. In addition to perturbing normal fiberogenesis, selenite treatment resulted in the disorganization of meridional rows. The authors proposed, "selenite blocks the cell cycle in the germinative zone of the lens epithelium in G_2 and/ or S phases" (42).

Selenite effects on metabolism

In order to understand the biochemical changes occurring in the lens in response to selenite, it is important to consider the metabolism of selenite. Selenite interacts chemically with sulfhydryl compounds, and is recognized as an active catalyst for GSH oxidation (43). Exposing hepatocytes to selenite (100uM), depleted thiols and increased the concentration of oxidized glutathione (44). The degree of glutathione oxidation in hepatocytes was a function of selenite concentration. The reduction of Se^{+4} to H_2Se (Se^{-2}) begins with the nonenzymatic reaction of selenite and GSH, with the subsequent formation of GSSG. The two intermediates of selenite metabolism, GSSeSG and GSSeH are substrates for glutathione reductase, and are reduced at the expense of NADPH (45). For every mole of selenite metabolized, 4 moles of GSH are oxidized, and 3 moles of NADPH are

consumed (45,37). The final product of metabolism, hydrogen selenide, may be methylated to form volatile derivatives. Selenite volatilization is inhibited when GSH is oxidized by hydroperoxide, or when gluconeogenesis is inhibited by p-tert-butylbenzoic acid (46). In vivo, dimethyl selenide, is excreted through the lungs, and trimethyl selenonium ion is excreted in the urine (47).

Selenite affects the properties of enzymes, and cellular organelles. Selenite is a potent stimulator of mitochondrial ATPase in rat liver (48) and an inhibitor of succinic dehydrogenase (49). Rounds, et al., found selenite caused mitochondrial swelling and fragmentation, inhibited oxygen consumption, and reduced cellular ATP content in rabbit endothelial cells and rat heart cells and (6). These authors proposed that selenite was acting as a mitochondrial uncoupler.

The influence of selenite on yeast cells was investigated by Potter and Elvehjem (7). Selenite treatment reduced oxygen uptake 80% when glucose, fructose, or mannose were used as substrate. However, oxygen uptake was not inhibited by selenite when lactate or pyruvate were used as substrates. These data are consistant with selenite as an inhibitor of glycolysis, rather than mitochondrial respiration.

Selenite affected membrane permeability in isolated rat hepatocytes. Anundi, et al. reported a concentration-dependent increase in cellular permeability upon treatment with selenite. Methionine was able to potentiate the effect of selenite in mediating cellular lysis. Electron micrographs of hepatocytes following selenite treatment revealed cell surface blebbing prior to the increase in cell permeability (45).

Biochemical changes accompanying selenite-cataract

Selenite-induced cataract is preceded by a series of biochemical changes in the lens. The incorporation of selenite into lenticular proteins changes water soluble and insoluble polypeptides (50). The insoluble protein fraction of the lens is increased in response to selenite, and is associated with 5-6 times more ^{75}Se relative to controls (51). Bhuyan, Bhuyan, and Podos have proposed that selenite-induced cataract results from conformational changes in structural and functional lens proteins (52).

Changes in lens thiol status occur in selenite-induced cataract. Bunce and Hess reported lens reduced glutathione decreased 60% 24 hours following an injection of sodium selenite (37). A concurrent elevation in oxidized glutathione, and 32% decrease in

lens NADPH concentration also occurred. Although NADPH is required to restore the pool of reduced glutathione, glutathione reductase was unaffected by selenite treatment (52). Reduced glutathione is important in protecting the lens from harmful oxidative reactions. Oxidation of lens protein thiols by selenite leads to disulfide bond formation, and selenotrisulfide cross linking, which compromises lens transparency (52).

Selenite impaired the lens' enzymatic defenses against oxidative damage. Bhuyan, Bhuyan, and Podos have found catalase and superoxide dismutase activities to decrease in lenses from animals given daily doses of selenite (52). Although glutathione peroxidase was not affected in the nuclear cataract, a 65% decrease in the enzyme was apparent in the mature selenite cataract. Hydrogen peroxide and malondialdehyde were elevated in ocular humors and lenses from selenite treated rats (52). Lipid peroxidation of the plasma membrane was also evident (52).

Calcium has been associated with lens opacification; human senile cataracts, X-ray cataracts, and selenite cataracts are accompanied by an accumulation of lens calcium (53, 54, 55). During cataract formation calcium is not redistributed, but is elevated in both the H_2O soluble and insoluble fraction (53). Bunce, Hess and

Batra reported a three-five fold elevation of lens calcium in selenite cataracts (55). Calcium has been implicated in the formation of lens HMW aggregates, and with a decrease of lens protein synthesis (56, 57). The function of calcium in selenite cataracts is uncertain.

Altered energy metabolism is one of the biochemical events leading to the formation of cataracts in the selenite treated animal. Tarnawska observed a 70% reduction in lens ATP concentration 24 hours following an injection of sodium selenite, with a concurrent elevation of lens AMP (5). Stimulation of glycolysis was manifested by an accumulation of lactate, a response consistent with the change in the ATP/AMP ratio. The effect of selenite on the TCA cycle in the lens was studied with 6-^{14}C-glucose. Virtually no $^{14}CO_2$ was released from lenses of control or injected animals, indicating pyruvate was converted to lactate rather than entering the TCA cycle (58).

Glutamine is another energy source for rat lens (59). At 24 hours following an injection of sodium selenite, lens oxidation of U-^{14}-C-glutamine to $^{14}CO_2$ decreased 27% (60). A greater affect of selenite on glutamine metabolism in the rat lens has been observed <u>in vitro</u>. (61)

The formation of selenite-induced cataract is an outward reflection of an internal perturbation in lens metabolism. A decrease in lens ATP concentration precedes the formation of cataract. This change in metabolism is critical to cell division, and the maintenance of lens ion fluxes, particularly Ca^{+2}. Elevated levels of oxidants, together with reductions in glutathione and enzymatic defenses make the lens more susceptible to damage. Finally, modifications in lens protein structure which occur in response to the selenite treatment, alter the refractive index of the lens, and decrease lens transparency.

III. EXPERIMENTAL PROCEDURES

Materials

Animals Sprague Dawley rats were obtained from Lab Animal Resources, VPI & SU. Female rats were housed with their litters in plastic cages, while breeder males were housed in stainless steel cages. All animals were maintained in a temperature controlled environment (21°C), on a twelve hour light cycle. Rats were supplied with commercial lab chow and distilled water ad libitum.

Reagents Hanks' 199 medium and fetal bovine serum were purchased from KC Biochemical (Lenexa, Kansas).

ATP disodium salt, benzylpenicillin (sodium salt), bovine serum albumin, DL-buthionine-(S,R)-sulfoximine,2-mercaptoethanol, dessicated firefly tails, 5,5'-dithiobis-2-nitrobenzoic acid, ethylenediamine tetraacetic acid disodium salt, glutathione reductase, glutathione, glycylglycine, imidazole, L-lactate lithium salt, lactate dehydrogenase, 2-p-iodophenyl-3-p-nitro-phenyl-5-phenyl tetrazolium chloride, phenazine methosulfate, tert-butyl hydroperoxide, sodium arsenate, sodium azide, and streptomycin sulfate were obtained from Sigma Chemical Company (St. Louis Missouri)

Sodium selenite and 2-vinylpyridine were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin).

Copper II chloride dihydrate and hydrochloric acid were purchased from J. T. Baker Chemical Company (Phillipsburg, New Jersey).

Standard analytical grade reagents were purchased from Fisher Scientific Company (Fair Lawn, New Jersey).

Instruments The following instruments were used in experimental and analytical procedurese:

LKB Ultrospec 4050 spectrophotometer, LKB Instruments Inc. (Gaithersburg, Maryland) interfaced with an Apple II e computer.

LKB 1217 Rackbeta scintillation spectrometer.

Psycotherm controlled environment incubator shaker, New Brunswick Scientific Company, Inc. (New Brunswick, New Jersey)

YSI Oxygen monitor,Yellow Springs Instrument Co., Inc. (Yellow Springd, Ohio)

Methods

In Vivo Experiments - A subcutaneous injection of 0.02M sodium selenite (30umol selenite/kg body weight) was given to ten-day old rat pups . In alternate experiments, an intraperitoneal injection of 0.4M DL-buthionine-(S,R)-sulfoximine was injected at 4 umol/g body weight. Control pups from the same litter were not injected. Rat pups were returned to their dams following an injection. Animals were decapitated, eyes were

rapidly removed from the ocular socket with care to minimize pressure on the eye as it is cut from the connective tissue. The eye was punctured with a scalpel at the optic nerve to release internal pressure from the vitreous humor. Lenses were removed through a large incision in the sclera and released onto filter paper which was pre-moistened with distilled water. Excess connective tissue was subsequently removed with a pair of forceps, and lenses were homogenized.

In Vitro Experiments - Eyes from appropriate age animals were immediately placed in modified Hanks' 199 medium. The eye was transferred to filter paper, and punctured a the optic nerve to release vitreous fluid. After cutting around the sclera, the lens was released into medium at 20-25^{o}C. Excess connective tissue was removed from the lens capsule with forceps. A lens pair was transferred to 2ml of fresh medium in a 25ml incubation flask, which was sealed with a rubber stopper subsequent to the addition of the lenses. The flask was flushed for ten seconds with 5% CO_2 air mixture, and incubated at 37^{o} C. Control incubation flasks contained 2.0ml modified Hanks' 199 medium, 0.1ml fetal bovine serum (and 0.21ml 95% ethanol with valinomycin experiments). In addition to control components, flasks

designated for treatment received .021ml of one (or combinations) of the following agents:

0.1M Sodium selenite

0.1M KCN

0.1M NaN_3

0.5M $CuCl_2$

0.5M t-BHP

10^{-5}M Valinomycin

Homogenate Preparation - Following the incubation, each lens was removed from its respective flask with a glass loop; excess medium was removed by gently blotting the lens on tissue. A 40% (w:v) homogenate was prepared with either 0.9M $HCLO_4$/0.05M H_3PO_4 or 0.1M TrisCl (pH 6.9), depending on assay conditions. Lenses were homogenized on ice. The homogenate was transferred to polypropylene microcentrifuge tubes and centreifuged at 8,000 x g for 5 minutes in an Eppendorf microcentrifuge. The supernatant was neutralized with 6.0 M K_2CO_3, and centrifuged 5 minutes at 8,000 x g. This final supernatant was stored at -20° C, and samples were assayed within a week.

Analytical Procedures

Determination of Adenosine-5′-triphosphate with Luciferase, adapted from Bernard L. Strehler (62)

Principle:

$$\text{ATP} + \text{Luciferin} \xrightarrow[Mg^{2+}]{\text{luciferase}} \text{Adenyl-luciferin} + \text{Pyrophosphate}$$

$$\text{Adenyl-luciferin} \xrightarrow{O_2} \text{Adenyl-oxyluciferin} + H_2O + \text{Light}$$

The fluorescent pigment luciferin, and the enzyme luciferase are extracted from firefly tails. In the presence of ATP, oxygen, and divalent cation the reaction procedes rapidly and results in the emmission of light. The quantity of ATP present in the sample is proportional to the intensity of light emitted. As ATP is consumed, or adenyl-oxyluciferin begins to accumulate, the reaction undergoes a first order decrease in rate.

Photon emission was quantified with a LKB Rackbeta 1217 scintillation spectrometer. Each sample was counted for three, twenty-second intervals to detect chemiluminescence. Lens ATP concentration was determined by linear regression from an ATP standard curve that was linear in the range of 20 to 400 picomoles of ATP.

Determination of Lens Lactate Production (63)

Principle:

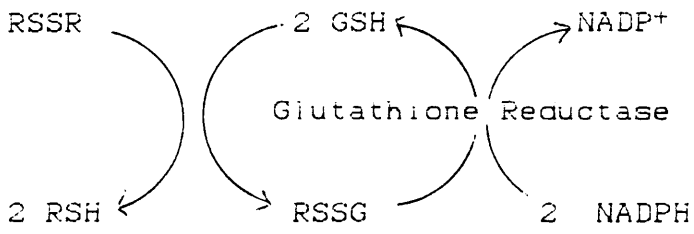
Lactate + NAD^+ lactate dehydrogenase pyruvate + NADH

Hydrazine Hydrazone

Lactate concentration was determined from the increase in absorbance at 340 nm due to the formation of NADH. The reaction is unfavorable in the forward direction, but the equilibrium can be displaced to the right by the removal of pyruvate via the hydrazine reaction (63). Lens lactate production was determined by assaying 0.2ml medium following the incubation of a lens pair at 37^0 C.

Total Glutathione Determination adapted from J. E. Brene and H. B. Burch (64)

Principle:



The glutathione assay was based on the ability of glutathione reductase to catalyze the oxidation of NADPH by the mixed disulfide Ellman intermediate (RSSG). The change in absorbance at 412 nm due to the accumulation of

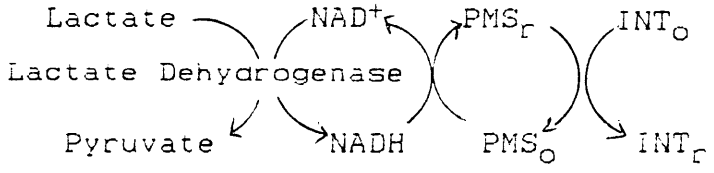
reduced Ellman compound (RSH), is followed over time. Lens glutathione concentration was determined from acidic extracts which were diluted 1:50 (v:v) with 5% sodium phosphate buffer. Values were extrapolated from a standard curve.

Hydrogen Peroxide Determination using Titanium IV adopted from B. D. Patterson, E. A. Macrae, I. B. Ferguson (65)

Lenses were frozen in liquid nitrogen and homogenized in 5% trichloroacetic acid. A complex of Ti (IV) with 4-(2-pyridylazo)resorcinol (PAR), is used to enhance the detection of H_2O_2 from the lens TCA extract. The absorbance at 508 nm was measured to detect H_2O_2; sample H_2O_2 concentrations were extrapolated from a standard curve which was linear between 10 and 50 nmoles of H_2O_2. The assay was modified to a one ml reaction volume in order to increase sensitivity.

Lactate Dehydrogenase Activity Determination (66)

Principle:



The reduction of NAD^+ is coupled to the reduction of the tetrazolium salt, 2-p-iodophenyl-3-p-nitrophenyl-5-

phenyl tetrazolium chloride (INT). Phenazine methosulfate serves as the intermediate electron carrier in the reaction. The extinction coefficient for INT at 503nm is $19.3 mM^{-1} cm^{-1}$ (66).

Oxygen uptake measurement (67)

Rat livers were homogenized in 0.25M sucrose, pH 7.4, and the mitochondrial fraction was isolated according to the method of Johnson and Lardy. Respiration was measured polarographically with a Clark electrode and YSI oxygen monitor. Experiments were conducted at 30°C, which provided an oxygen content of 237 nmoles O_2/ml of air saturated water.

IV. RESULTS

The effect of selenite on lens ATP in vivo

A single, subcutaneous injection of sodium selenite (30 umol/kg body weight) in 10 day old rats produced nuclear cataracts within 72 hours. The lens opacification is accompanied by altered ATP levels. Within the first 48 hours post-injection, fluctuating ATP concentration occurred in control and injected animals. Within the next 24 hours, the control ATP concentration per lens became constant, while the lenses from injected animals showed a steady decline in ATP (figure 1). By 11 days following a selenite injection, lens ATP was reduced by 45%.

The decrease in lens ATP was examined relative to the increase in lens weight with age. Lens growth in selenite injected animals was surpressed compared to control lenses. Figure 2 illustrates the selenite effect when lens ATP is expressed according to lens weight. Fluctuation in ATP in lenses from control and injected animals occurred during the first 36 hours. By 48 hours post-injection control lenses sustained a constant ATP level while ATP in lenses from injected animals diverged from control values. By 11 days post-injection ATP concentration between control and treated lenses differed

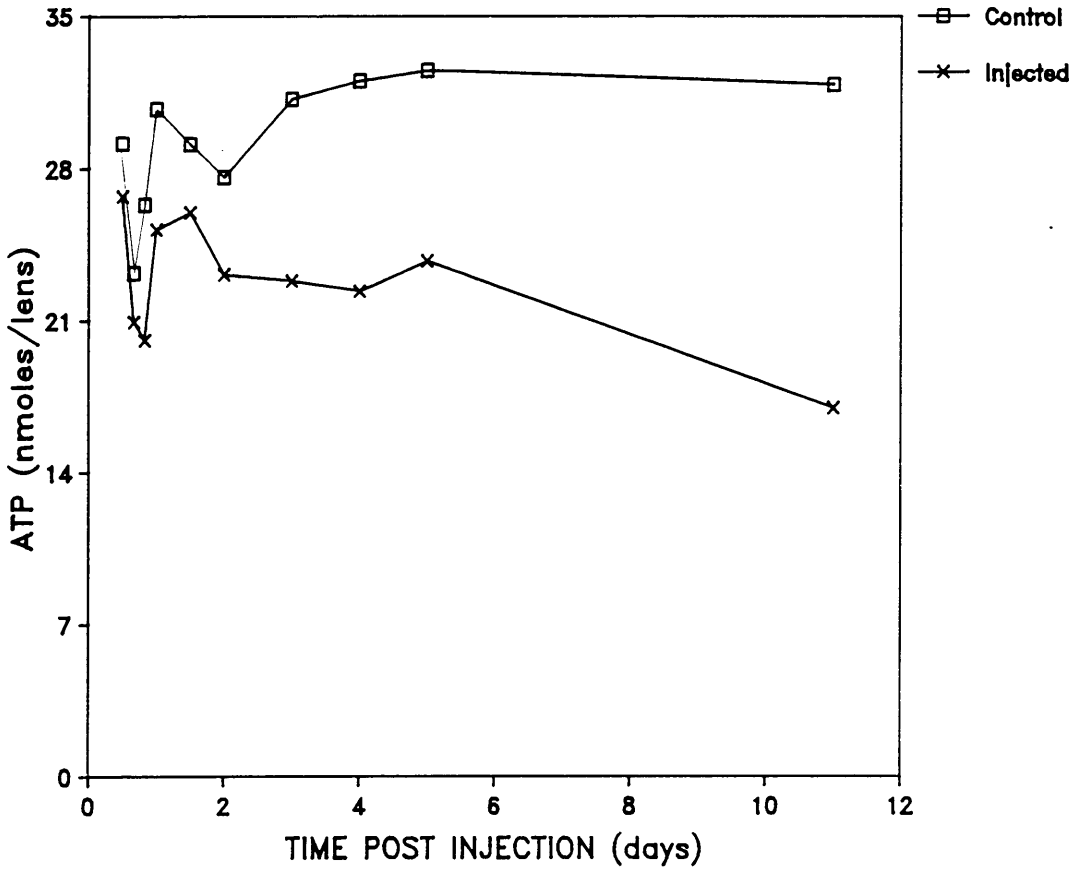


Figure 1. Comparison of rat lens ATP content in control and selenite injected animals

A single subcutaneous injection of sodium selenite (30 umol/kg body weight) was administered to ten 10-day old rats. Control litter mates were non-injected. Lenses were isolated at various time points following an injection, and homogenized in 0.05M H_3PO_4/0.9M $HCLO_4$ (1:40, w:v). Neutralized lens extracts were analyzed for ATP by a luciferase assay. Data are expressed on a per lens basis.

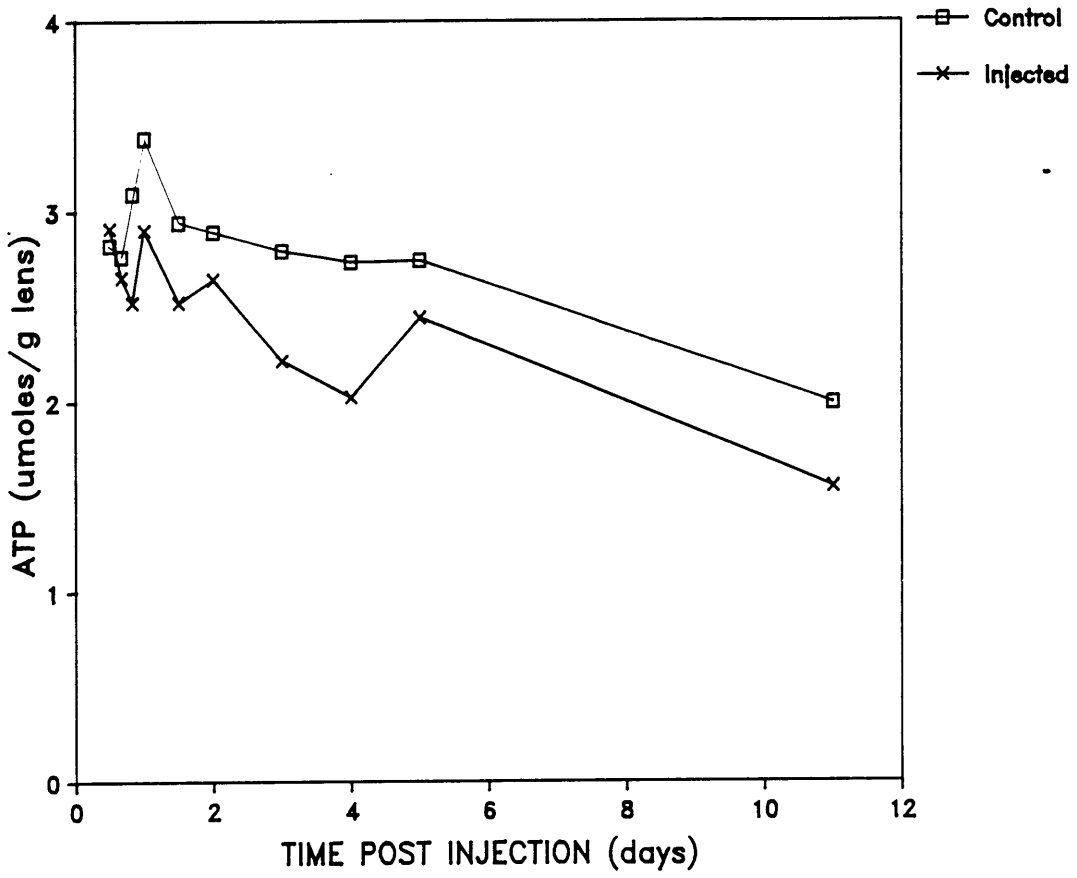


Figure 2. Comparison of rat lens ATP concentration from control and selenite injected animals

A single subcutaneous injection of sodium selenite (30 umol/kg body weight) was administered to ten 10-day old rats. Control litter mates were non-injected. Lenses were isolated at various time points following an injection, and homogenized in 0.05M H_3PO_4/0.9M $HCLO_4$ (1:40, w:v). Neutralized lens extracts were analyzed for ATP by a luciferase assay. Lens ATP concentration was determined at various time points following a selenite injection. Data are expressed according to lens wet weight.

by only 22%.

The overall relationship between lens weight and ATP concentration/g lens was defined by a correlation coefficient of -0.788 for control lenses based on 154 data points. For lenses from injected animals the correlation coefficient of -0.796 defined this same relationship based on 153 data points. A moderate overall correlation existed between lens weight and total ATP content in control lenses (correlation coefficient 0.509) but this relationship disappeared with selenite treatment (correlation coefficient 0.0228). When one compared the correlation of total lens ATP with lens age the correlation coefficients were 0.458 and -0.5224 for the control and treated lenses respectively. Primary analysis of the data identifies the significant effect of selenite treatment on decreasing lens ATP content.

The data consisted of unbalanced cells and were run as a general linear model ANOVA using 153 control values and 154 treated values. Comparison between control and treatments indicated a significant treatment effect for both ATP concentration and total lens ATP at the 95% level.

The effect of selenite on ATP in vitro

In vitro, 1mM sodium selenite produced a steady decline in lens ATP over a 24 hour period (Figure 3). After incubating 4 hours with selenite, lens ATP concentration was reduced 15%, and reached 20% of control values within 24 hours. A peach colored precipitate on the lens surface resulted from selenite reduction products and an increased frequency of lens blebbing occurred over 24 hours. Incubation for 24 hours imposed a stress on control lenses as well; ATP from control lenses was reduced approximately 30%. The effect of selenite on energy producing pathways in the lens was compared to effects produced by metabolic inhibitors. Selenite treatment in vitro resulted in a 10% decrease in lens lactate production (Figure 4). Lens lactate production was inhibited 40% with $CuCl_2$ and 15% with t-BHP; this effect is enhanced by the addition of selenite (Figure 5). In contrast, lactate production was elevated in lenses exposed to azide and KCN.

Selenite reduced lens ATP 15% during a 4 hour incubation (Figure 6). Lens ATP decreased when KCN, azide, valinomycin, $CuCl_2$ or t-BHP were in the culture medium. A concentration dependent decrease in ATP content occurred with up to 1.0mM $CuCl_2$. ATP decreased 21% at 1.0mM azide and 30% at 5.0mM azide. Selenite used

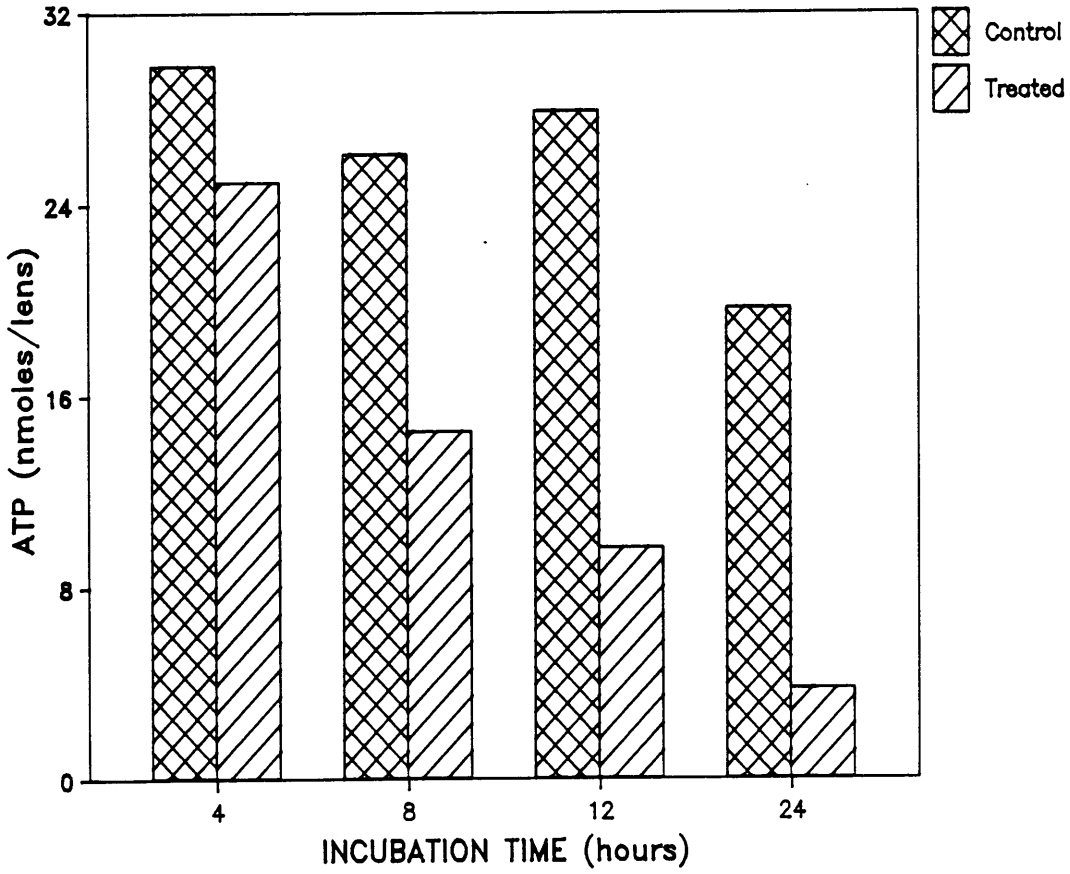


Figure 3. Comparison of lens ATP concentration as a function of selenite exposure time

Lenses from 10-day old rats were cultured 4 hours in modified Hanks' medium TC 199 with 5% fetal bovine serum, at 37°C. Treatment included the addition of 1mM Na_2SeO_3 to the culture medium. After different incubation intervals, a lens homogenate was prepared with 0.05M H_3PO_4/0.9M $HCLO_4$ (1:40, w:v). Lens ATP concentration was determined from neutralized lens extracts by a luciferase assay.

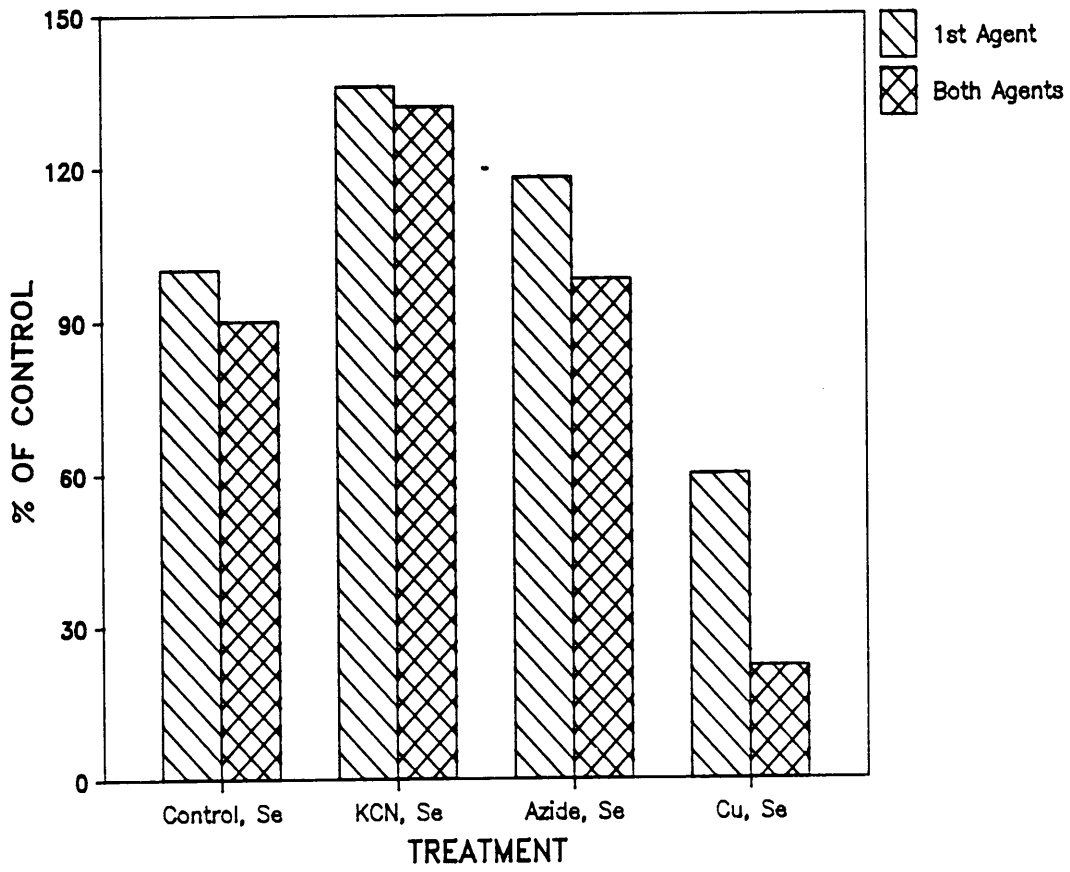


Figure 4. Comparison of lens lactate production in vitro

Lenses from 10-day old rats were cultured 4 hours in modified Hanks' medium TC 199 with 5% fetal bovine serum, at 37°C. In addition, treated lenses contained inhibitors in the medium: Se (1.0mM Na_2SeO_3), KCN (1.0mM KCN), Azide (1.0mM NaN_3), and Cu (0.5mM $CuCl_2$). Lens lactate production (umoles/ml), in the presence of inhibitors was quantified from the medium, and is presented as a percentage of the control.

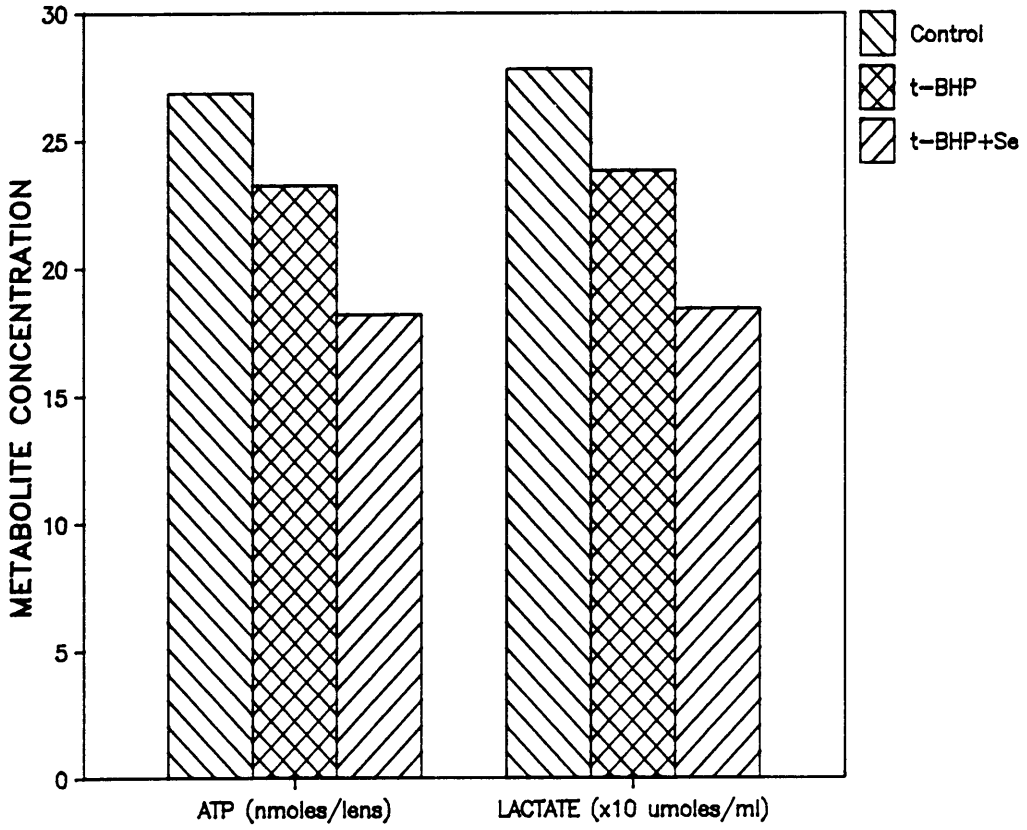


Figure 5. The effect of t-BHP on lens lactate production and ATP concentration

Lenses from 10-day old rats were cultured 4 hours, at 37°C in Hanks' medium TC 199 and 5% fetal bovine serum. Treated lenses were exposed to 0.5mM t-BHP in the presence or absence of 1.0mM Na_2SeO_3. Following the incubation period, lens ATP concentration was determined from a 40% lens homogenate prepared with 0.05M H_3PO_4/0.9M $HClO_4$. Lens lactate production was quantified from the medium.

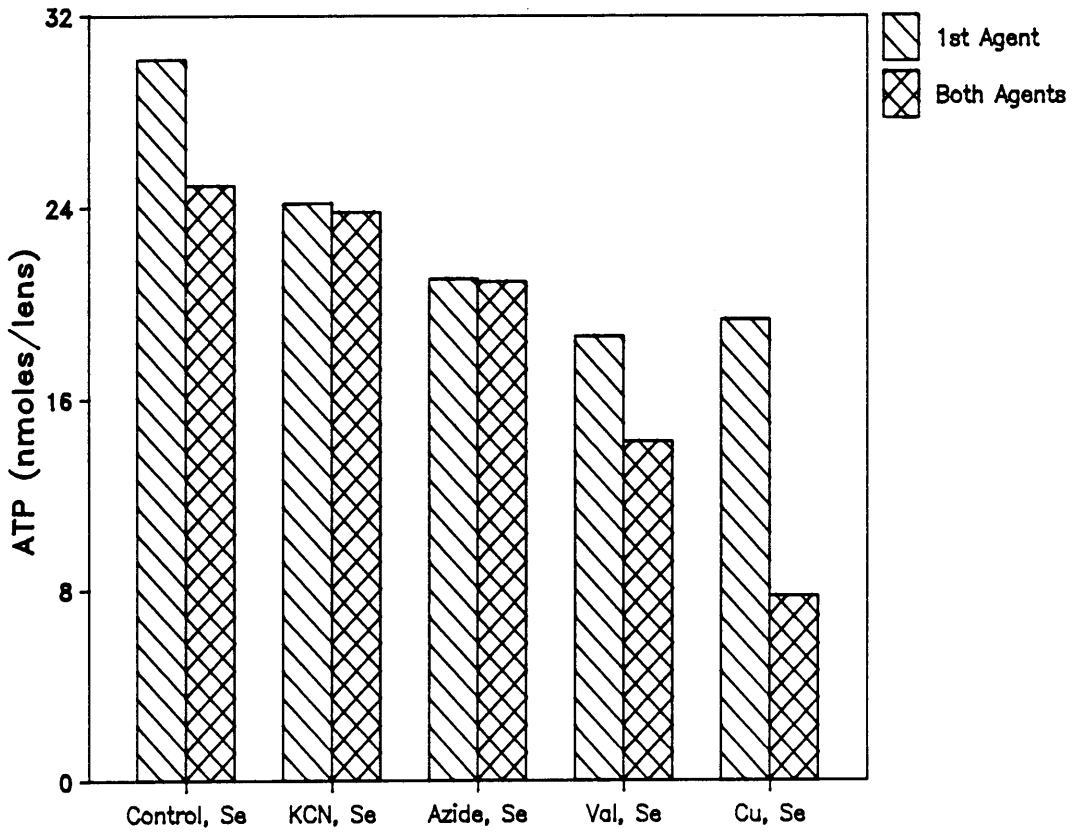


Figure 6. The relative effects of selenite and inhibitors of energy metabolism on lens ATP content

Lenses from 10-day old rats were cultured 4 hours in modified Hanks' medium TC 199 with 5% fetal bovine serum, at 37°C. Inhibitors were added to the medium in the presence or absence of sodium selenite; Se (1.0mM Na_2SeO_3), KCN (1.0mM KCN), azide (1.0mM NaN_3), Cu (0.5mM $CuCl_2$), and val ($1x10^{-2}$mM valinomycin). Following the incubation, lenses were homogenized in 0.05M H_3PO_4/0.9M $HCLO_4$ (1:40, w:v) and analyzed for ATP by the luciferase assay. Duncan's Multiple Range Test (alpha .05) indicated treatments with Se, Cu, and val are statistically different from controls.

concurrently with t-BHP, valinomycin or $CuCl_2$ potentiated the drop in ATP (Figures 5 & 6). This enhanced effect did not occur when selenite was used together with KCN or azide .

The relationship of selenite to the inhibitors used throughout these studies is represented in Figures 7-10. The complementary effect of selenite and t-BHP on lens ATP resembles the change in ATP observed by treatment with t-BHP and azide. Compared to treatment with t-BHP alone, combined treatments with selenite or azide resulted in a 21% and 20% decrease in lens ATP, respectively (Figure 7). The interaction of selenite with $CuCl_2$ resulted in a synergistic reduction in ATP which is not represented by any other treatments (Figure 8). When selenite and azide were present together in the medium there was no further decrease in lens ATP. In contrast, $CuCl_2$ or t-BHP decreased lens ATP further than treatment with azide alone (Figure 9). The decrease in lens ATP varied with valinomycin treatment (Figure 10). A complementary decrease in lens ATP occurred with valinomycin and either selenite, azide, $CuCl_2$, or t-BHP (Figure 10).

Oxygen uptake experiments with rat liver mitochondria used 3-5 mg mitochondrial protein, 3.3mM substrate, 0.3mM ADP and 1.0mM sodium selenite. Typical

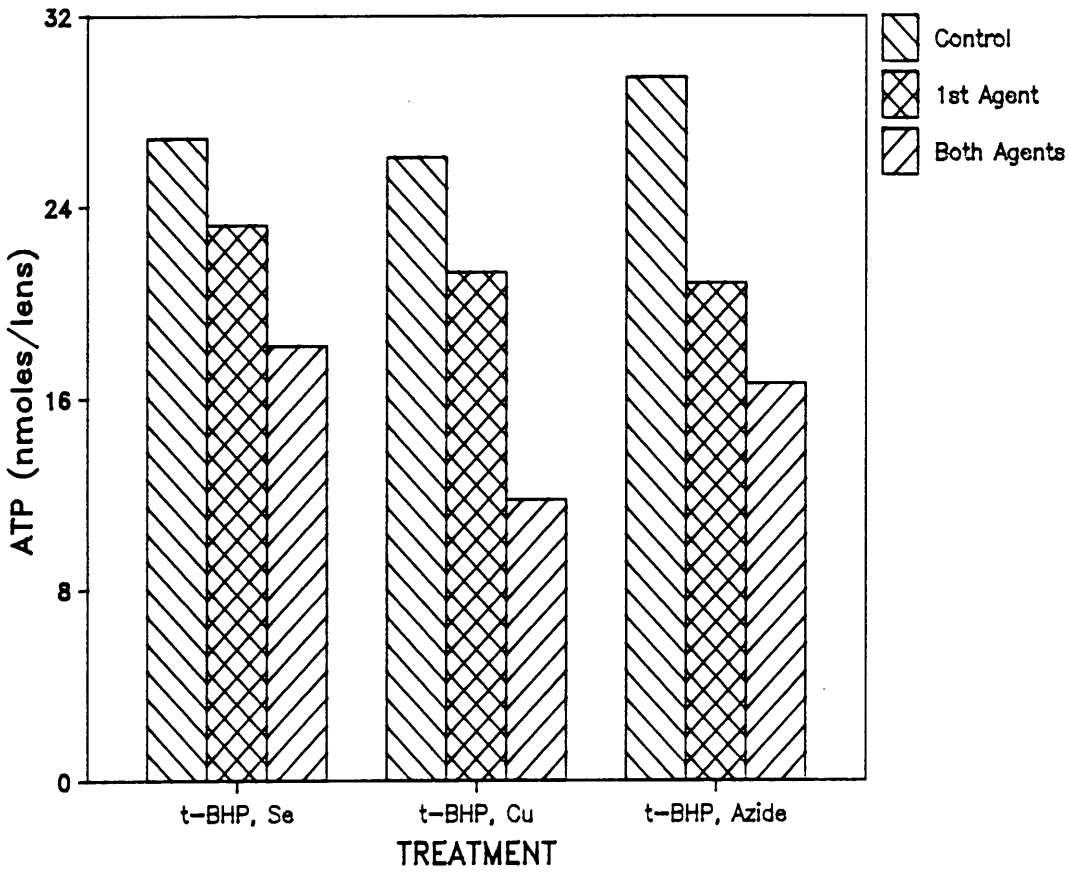


Figure 7. Effect of t-BHP and other inhibitors on lens ATP concentration

Lenses from 10-day old rats were cultured 4 hours, at 37°C in Hanks' medium TC 199 and 5% fetal bovine serum. Treated lenses were exposed to 0.5mM t-BHP in the presence or absence of 1.0mM Na_2SeO_3, 0.5mM $CuCl_2$, or 1.0mM NaN_3, respectively. A 40% lens homogenate was prepared with 0.05M H_3PO_4/0.9M $HClO_4$, and analyzed for ATP content by a luciferase assay. In treatments with two agents, concentrations were found to be significantly different from concentrations with single agent treatments by Duncan's Multiple Range Test (alpha = .05). All treatments were lower than control values.

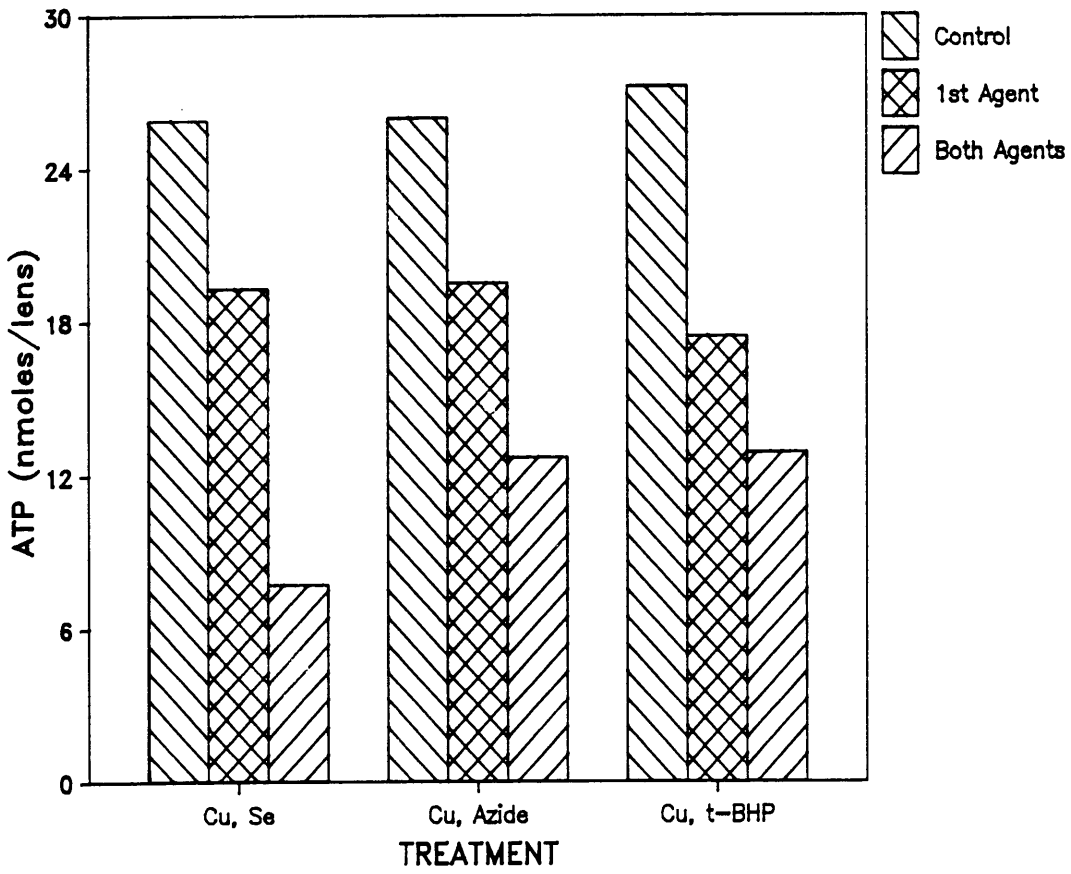


Figure 8. Effect of Copper and Inhibiors on lens ATP concentration

Lenses from 10-day old rats were cultured 4 hours, at 37°C in Hanks' medium TC 199 and 5% fetal bovine serum. In addition, treatments included 0.5mM $CuCl_2$ in the absence or presence of 1.0mM Na_2SeO_3, 1.0mM NaN_3, or 0.5mM t-BHP. A 40% lens homogenate was prepared with 0.05M H_3PO_4/0.9M $HClO_4$, and analyzed for ATP content by a luciferase assay. In treatments with two agents, concentrations were found to be significantly different from concentrations with single agent treatments by Duncan's Multiple Range Test (alpha = .05). All treatments were lower than control values.

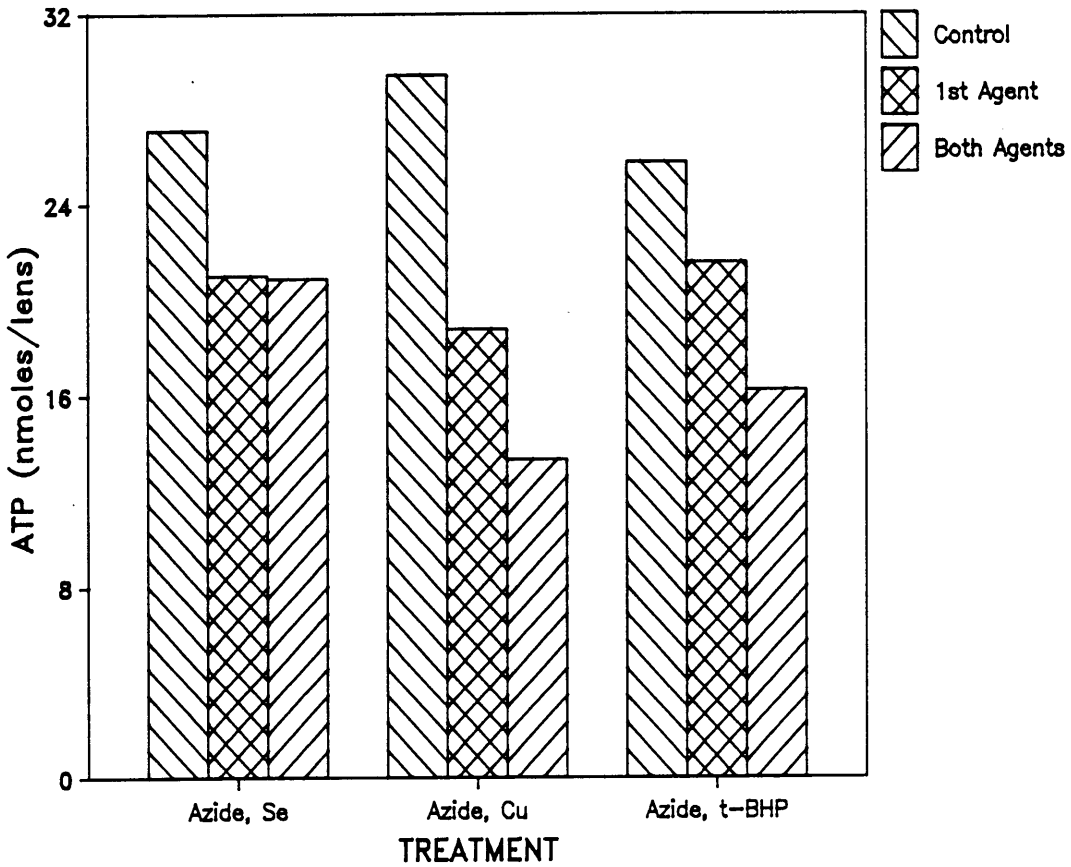


Figure 9. Effect of sodium azide and other inhibitors on lens ATP concentration

Lenses from 10-day old rats were cultured 4 hours, at 37°C in Hanks' medium TC 199 and 5% fetal bovine serum. In addition, treatments included 1.0mM NaN_3 in the presence or absence of 1.0mM Na_2SeO_3, 0.5mM $CuCl_2$, or 0.5mM t-BHP, respectively. A 40% lens homogenate was prepared with 0.05M H_3PO_4/0.9M $HClO_4$, and analyzed for ATP content by a luciferase assay. For all treatments concentrations were significantly different from controls, and concentrations were significantly different for combined treatments of azide with either $CuCl_2$ or t-BHP by Duncan's Multiple Range Test (alpha = .05).

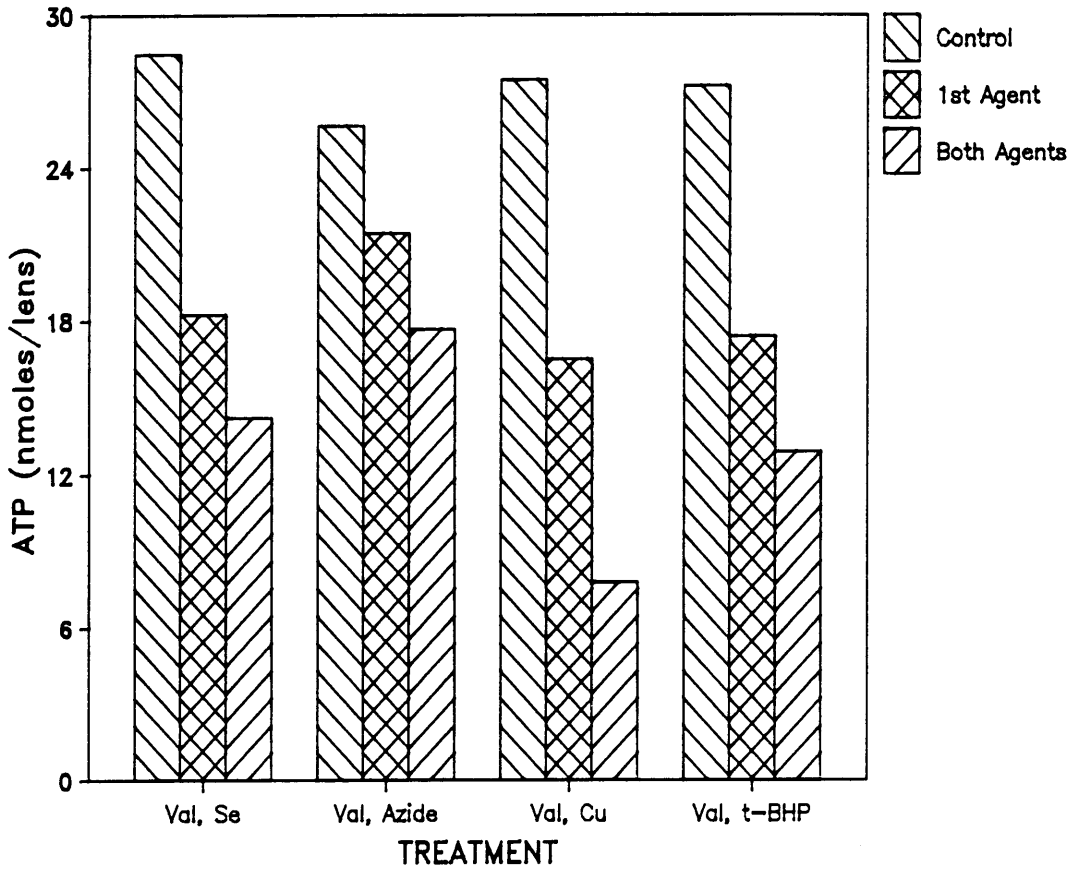


Figure 10. Effect of valinomycin and other inhibitors on lens ATP concentration

Lenses from 10-day old rats were cultured 4 hours, at 37°C in Hanks' medium TC 199 and 5% fetal bovine serum. In addition, treatments included $1x10^{-2}$mM valinomycin in the presence or absence of 1.0mM Na_2SeO_3, 1.0mM NaN_3, 0.5mM $CuCl_2$, or 0.5mM t-BHP, respectively. A 40% lens homogenate was prepared with 0.05M H_3PO_4/0.9M $HClO_4$, and analyzed for ATP content by a luciferase assay. In treatments with two agents, concentrations were found to be significantly different from concentrations with single agent treatments by Duncan s Multiple Range Test (alpha = .05). All treatments were lower than control values.

O_2 uptake rates (umol/min/mg protein) varied between .04-.05 with succinate and was approximately .015 with alpha-ketglutarate. Selenite inhibited oxygen uptake when alpha-ketoglutarate, glutamate, and malate were used as substrates. With succinate as substrate, virtually no effect on oxygen uptake was observed (Table I). The addition of succinate following selenite inhibition alleviated the selenite inhibition.

The effect of selenite on lens glutathione

Selenite is known to oxidize glutathione and utilize NADPH reducing equivalents as it is metabolized to volatile derivatives. Treatment with 1mM selenite during a 4 hour incubation reduced lens glutathione 25% from controls (Figure 11). Glutathione decreased 25% and 15%, respectively when lenses were treated with t-BHP or $CuCl_2$. The reduction in lens glutathione by these agents together was additive (Figure 12). Azide and valinomycin had no effect on lens glutathione content (Figures 11,13).

The impact of BSO (buthionine-sulfoximine), a specific inhibitor of gamma-glutamylcysteinyl synthetase, on lens metabolism is shown in Figure 14. An single intraperitoneal injection of BSO (10umol/g body weight) to 10 day old rats decreased lens glutathione 90%, 49 hours following the injection. No glutathione was

TABLE I.

Rat liver mitochondria were isolated in 0.25M sucrose. 3-5mg of mitochondrial protein were monitored for oxygen uptake using a Clark electrode and a YSI biological oxygen monitor following the addition of 3.3mM substrate, 0.3mM ADP and 1.0mM Na_2SeO_3.

SUBSTRATE	% INHIBITION BY Na_2SeO_3
α-Ketoglutarate	67
Glutamate	44
Malate	62
Succinate	nd[a]

[a] Not detectable

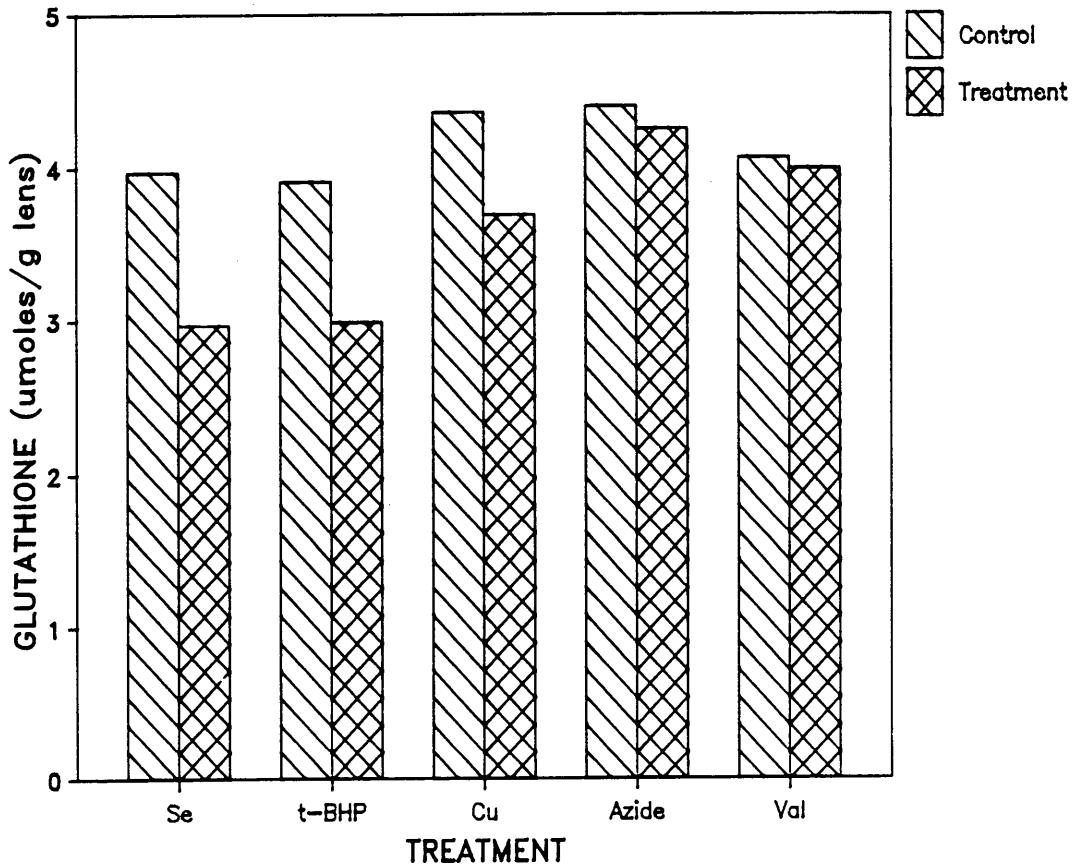


Figure 11. Total glutathione of the neonate rat lens in vitro

Lenses from 10-day old rats were incubated 4 hours, at 37°C in Hanks' medium TC 199 and 5% fetal bovine serum in the presence or absence of 1.0mM Na_2SeO_3, 0.5mM t-BHP, 0.5mM $CuCl_2$, 1.0mM NaN_3, or $1x10^{-2}$mM valinomycin, respectively. Following the incubation, lenses were homogenized with 0.05M H_3PO_4/0.9M $HClO_4$ (1:40, w:v), and the acidic extract was analyzed for glutathione.

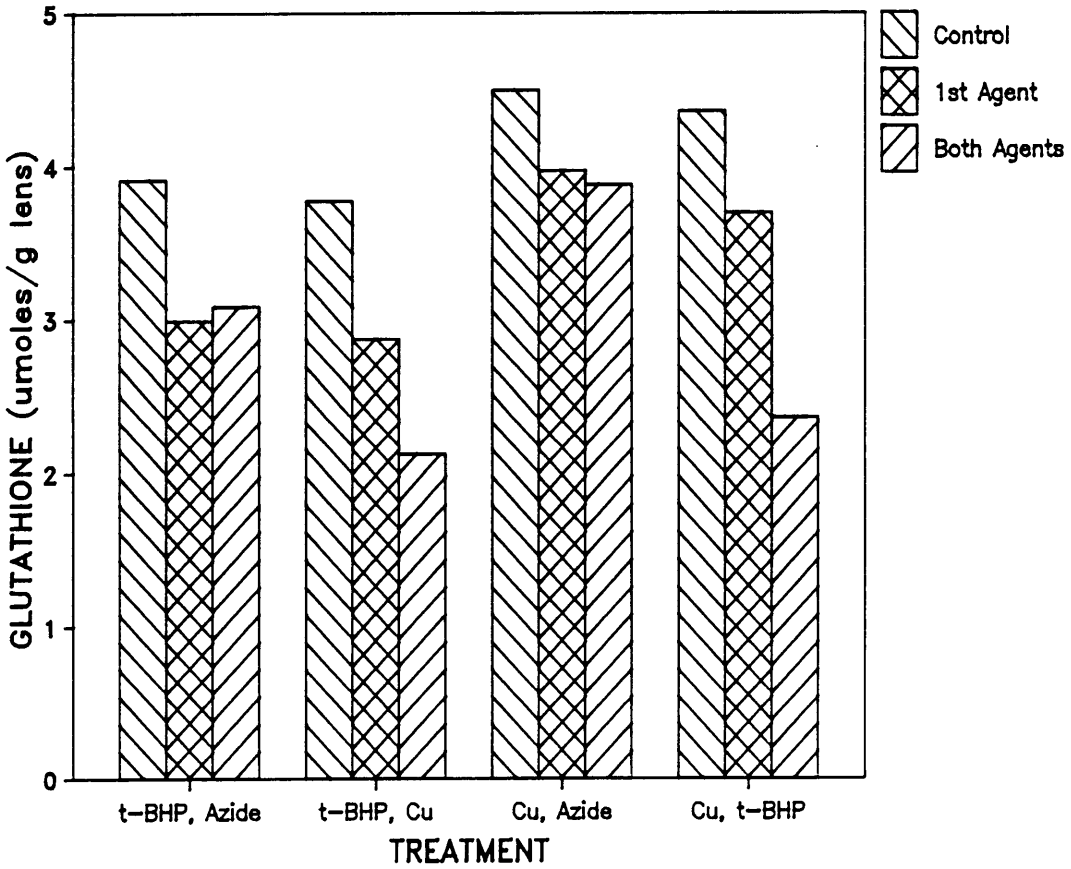


Figure 12. The effect of inhibitors on lens total glutathione

Lenses from 10-day old rats were incubated 4 hours, at 37°C in Hanks' medium TC 199 and 5% fetal bovine serum. Medium for treatments contained 0.5mM t-BHP in the absence or presence of , 1.0mM NaN_3 or 0.5mM $CuCl_2$ or, 0.5mM $CuCl_2$ in the presence or absence of 1.0mM NaN_3 or 0.5mM t-BHP, respectively. Following the incubation, lenses were homogenized with 0.05M H_3PO_4/0.9M $HClO_4$ (1:40, w:v), and the acidic extract was analyzed for glutathione.

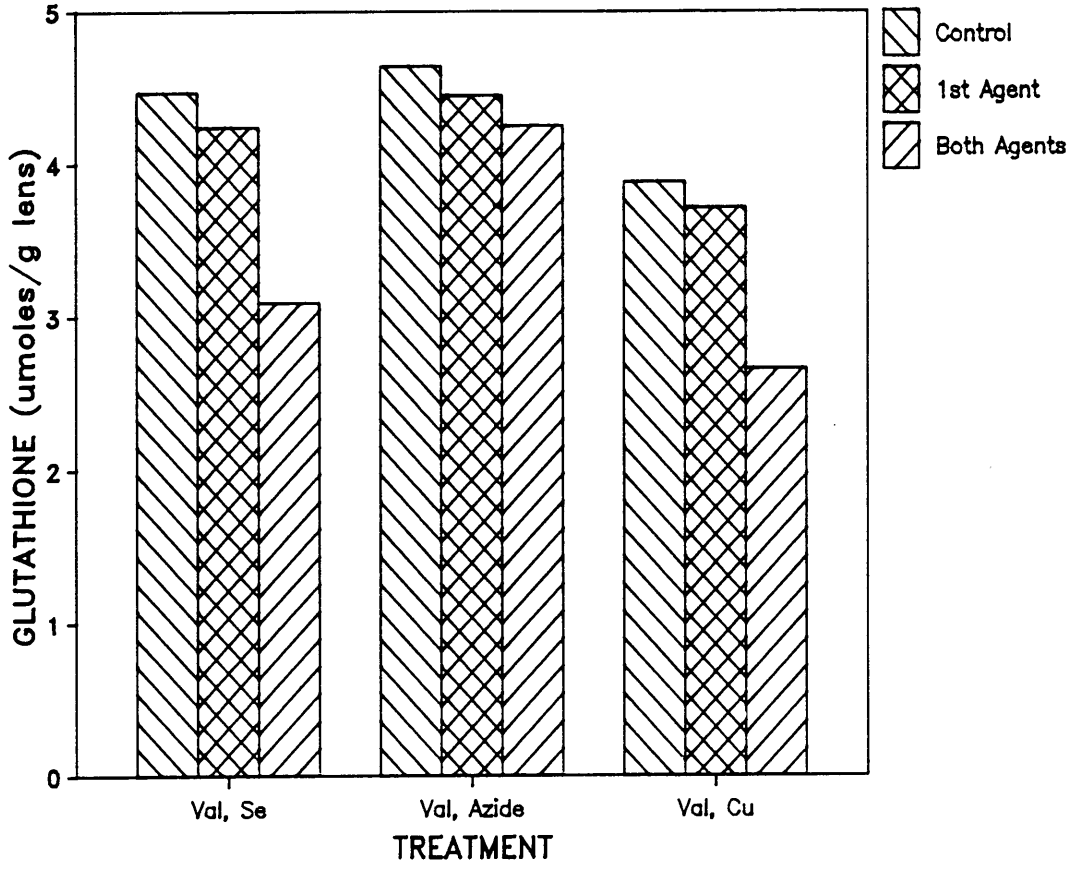


Figure 13. Total glutathione in valinomycin treated lenses

Lenses from 10-day old rats were incubated 4 hours, at 37°C in Hanks' medium TC 199 and 5% fetal bovine serum. Medium for treatments contained $1x10^{-2}$mM valinomycin in the presence or absence of 1.0mM Na_2SeO_3, 1.0mM NaN_3, or 0.5mM $CuCl_2$, respectively. Following the incubation, lenses were homogenized with 0.05M H_3PO_4/0.9M $HClO_4$ (1:40, w:v), and the acidic extract was analyzed for glutathione.

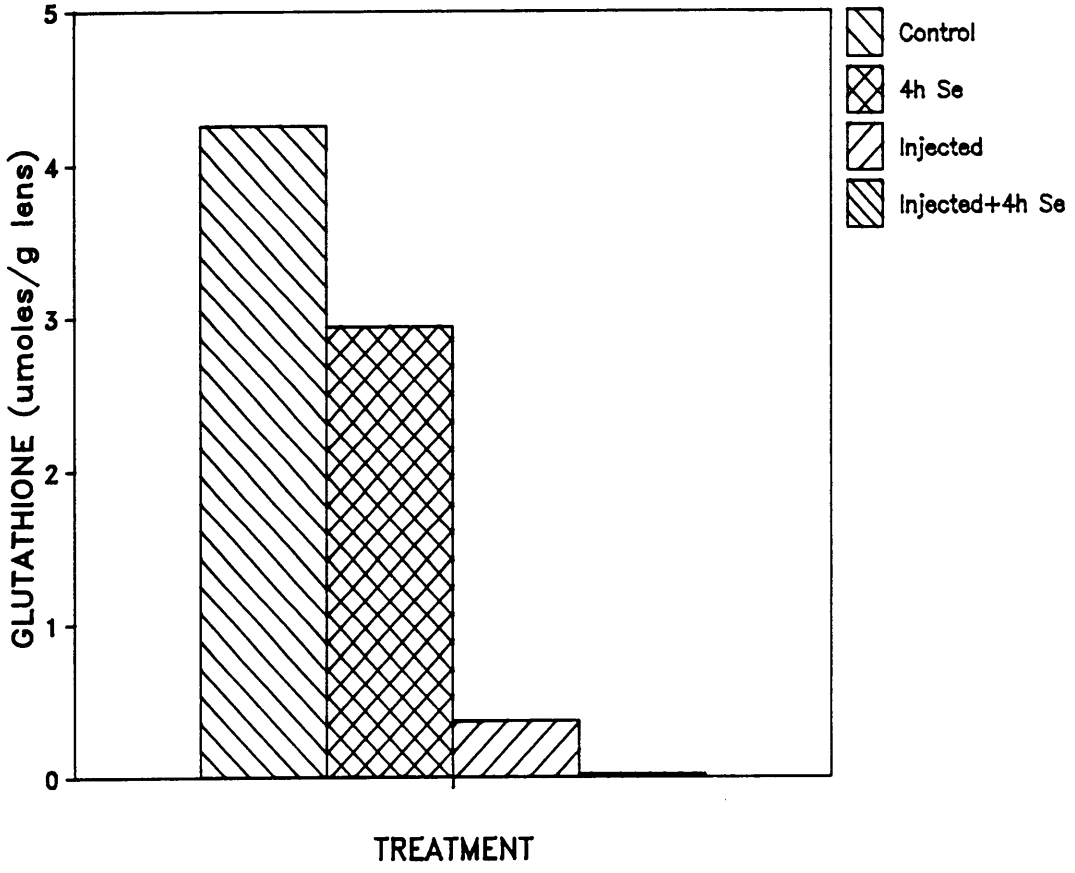


Figure 14. Total glutathione in control and BSO injected animals

Rat lenses were isolated 49 hours following an intraperitoneal injection of (BSO) buthionine-sulfoximine (4 umol/g body weight) to 10-day old animals. Control litter mates were not injected. Half of the lenses from control and injected animals were subsequently incubated 4 hours at 37°C, in Hanks' medium TC 199 and 5% fetal bovine serum. Lenses were treated in vitro with 1mM Na_2SeO_3. Following the incubation, lenses were homogenized with 0.05M H_3PO_4/0.9M $HClO_4$ (1:40, w:v), and the acidic extract was analyzed for glutathione.

detected when lenses from BSO injected animals were subsequently incubated 4 hours in Na_2SeO_3. BSO had no affect on lens ATP concentration when it was in the incubation medium, nor when it was injected into rats (Table II). However, selenite had reduced lens ATP following a BSO injection.

Lenses incubated with azide or selenite were analyzed for hydrogen peroxide content. The data presented in Table III indicate no differences in the hydrogen peroxide concentration between control and treated lenses.

The effect of selenite on lactate dehydrogenase leakage

The effect of selenite on lens permeability was investigated by measuring lactate dehydrogenase leakage from the lens (Figure 15). Data reveal a difference in lens lactate dehydrogenase leakage between selenite treatment *in vivo* and *in vitro*. Lens permeability 24 hours following an injection of selenite does not seem to be affected, while lenses treated with selenite in culture released nearly twice as much lactate dehydrogenase as control lenses. Treatment with *t*-BHP, a general oxidant, resulted in a 3-fold elevation of lactate dehydrogenase activity in the medium. Together,

TABLE II.

The effect of buthionine-sulfoximine on lens ATP

Rat lenses were isolated 49 hours following an intraperitoneal injection of BSO[a] to 10-day rat pups. Half of the lenses from control and injected animals were subsequently treated 4 hours with 1mM Na_2SeO_3 *in vitro*. In a second experiment, lenses from 10 day old rats were incubated 4 hours in the presence of 1mM BSO. Lenses were homogenized in 0.9M $HClO_4$/0.05M H_3PO_4 (1:40, w:v), and lens ATP was quantified by a luciferase assay.

Treatment	ATP[b] (nmol/lens) Control	Treated
In vivo		
49 h PI[c]	29.47 ± 0.87	30.82 ± 1.60
49 h PI + 4h SeO_3^{-2}	22.67 ± 2.27	22.00 ± 1.13
In vitro		
4 h Incubation in BSO	28.36 ± 0.71	28.62 ± 0.58

[a] 4 umol buthionine-sulfoximine/g body weight

[b] Values expressed as mean ± standard error

[c] Hours post injection

TABLE III.

Hydrogen Peroxide in the Lens

Lenses from 10-day old rats were incubated in modified Hanks' medium TC 199 at 37°C in the presence of 1mM NaN_3 or 1mM Na_2SeO_3. Lenses were homogenized in 5% TCA. H_2O_2 was quantified by a colorimetric assay using $TiCl_4$.

Incubation Time (hours)	Treatment	H_2O_2[a] umol/glens	nmol/lens
4	Control	3.68 ± 0.4	37.87 ± 1.1
	NaN_3	3.72 ± 0.2	37.53 ± 1.2
6	Control	3.91 ± 0.5	38.84 ± 4.8
	Na_2SeO_3	3.47 ± 0.3	36.67 ± 4.0

[a] Values represent mean ± standard errror

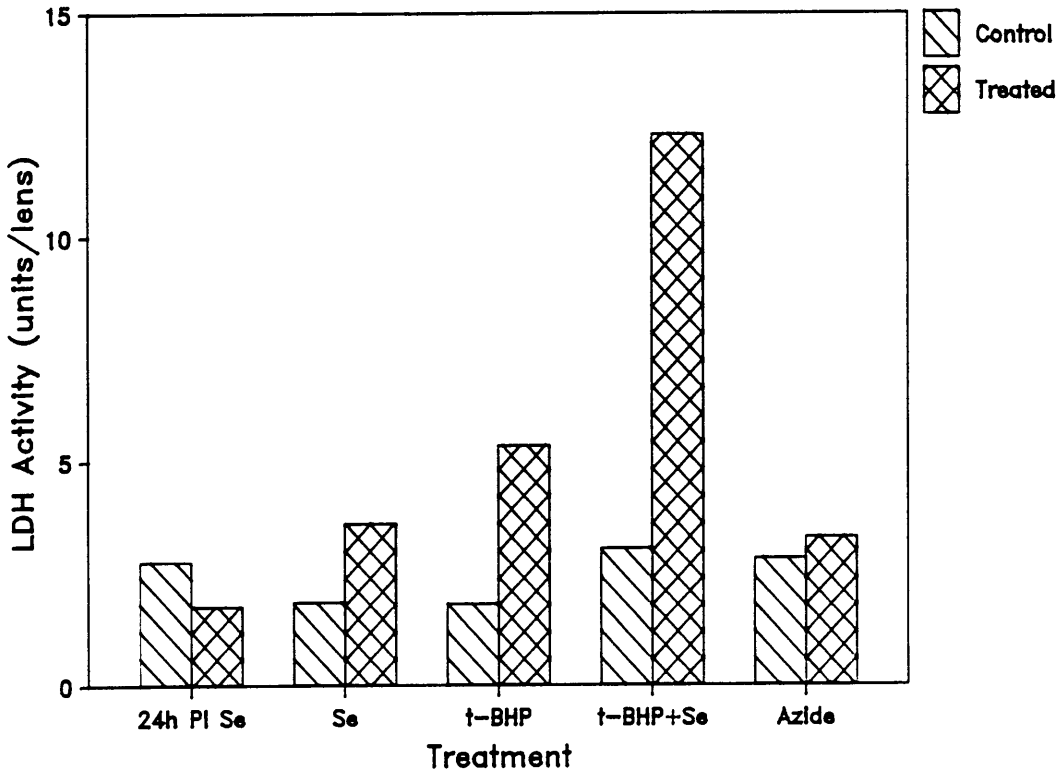


Figure 15. Lens lactate dehydrogenase leakage

In the first treatment, a subcutaneous injection of Na_2SeO_3 was administered to 10-day old rats. Lenses were isolated 24 hours post injection and incubated 6 hours, 37°C, in Hanks' medium TC 199 and 5% fetal bovine serum. In the remaining experiments, lenses from 10-day old rats were exposed to inhibitors under the described culture conditions: 1.0mM Na_2SeO_3, 0.5mM t-BHP, 1.0mM Na_2SeO_3 and 0.5mM t-BHP, or 1.0mM NaN_3, respectively. LDH activity (1 unit = 1 nmole/min) represents the enzyme activity detected in the culture medium.

selenite and t-BHP produced a 4-fold elevation in lactate dehydrogenase released into the medium. Azide had a marginal effect on lactate dehydrogenase leakage. Table IV lists the percent of total lens lactate dehydrogenase released into the incubation medium.

TABLE IV.

Lens lactate dehydrogenase activity

Lens membrane permeability was evaluated by quantifying lactate dehydrogenase leakage from lens pairs following a 6 hour incubation in 0.5ml modified Hanks' medium TC 199 at 37°C. LDH activity was determined by a colorimetric assay with INT.

Treatment	Medium Activity (units/lens)[a]	Lens Activity (units/lens)[a]	%LDH Leakage
Control	2.76 ± 0.36	59.03 ± 6.24	4.5
24h PI SeO_3^{-2}	1.75 ± 0.08	56.33 ± 4.61	3.0
Control	1.85 ± 0.10	86.93 ± 2.33	2.1
SeO_3^{-2}	3.60 ± 0.83	100.26 ± 1.93	3.5
Control	1.81 ± 0.32	50.48 ±13.47	3.5
t-BHP	5.34 ± 0.50	64.95 ± 5.11	7.6
Control	3.05 ± 0.23	66.11 ± 2.37	4.4
t-BHP + SeO_3^{-2}	12.30 ± 1.22	45.40 ± 2.00	21.3
Control	2.83 ± 0.59	71.23 ± 2.86	3.8
Azide	3.29 ± 0.53	65.99 ± 6.00	4.7
Control	2.37 ± 0.34	-	-
$CuCl_2$	nd[b]	-	-
Control	2.63 ± 0.60	-	-
$CuCl_2$ + SeO_3^{-2}	nd	-	-

[a] units/lens x 10^3, values represent mean ± standard error

[b] not detected

V. DISCUSSION

IN VIVO RESPONSES

A primary objective was to describe accurately the response of ATP concentration in rat lenses of animals treated with a single, subcutaneous injection of sodium selenite. A 27% decrease in total lens ATP had been reported to occur 24 hours following a subcutaneous injection of selenite and lens ATP content recovered by 96 hours post injection (5). The present research confirmed a reduction in lens ATP content following selenite treatment, but, the effect on ATP content was quantitatively less.

The selenite injection is administered during a critical period for lens maturation in the rat, 10-14 days of age (68). Cataract formation, caused by selenite, is limited to this early, postnatal, developmental period (40). The total ATP is stabilized in control lenses after 14 days (Figures 1 & 2). Fluctuations in ATP content, between days 10 and 13, may be related to changes in a high demand for ATP during this developmental period. Lens sensitivity to selenite at days 10-12 may reflect the significance of this increasing ATP pool. Any impact on ATP content before the tissue has reached a steady state concentration of ATP can result in significantly altered metabolism.

The *in vivo* ATP phenomenon has been examined as total content of ATP in the lens, and as ATP concentration per gram tissue. Obviously ATP content is less in lenses from injected animals (Figure 1). However, the difference in ATP concentration between control and treated samples is smaller (Figure 2). The difference between figures 1 and 2 reflects the slower growth rate of the lens in animals treated with selenite which has been reported (37). Both ATP content and lens weight correlate with lens age. Lens growth supressed by selenite may reflect compromised protein synthesis. When expressing ATP concentration per gram tissue, the greater mass of a lens results in a concentration decrease over time as a consequence of lens fiber cell maturation. The normal increase in lens weight is derived from lens hydration and an increase in protein, while the amount of physiological machinery for ATP synthesis remains constant (8). It was concluded that selenite treatment significantly reduces lens ATP concentration and that this effect is magnified by the sustained inhibition of lens growth. Furthermore, lenses from selenite treated animals were unable to recover from the effect on the ATP pool.

IN VITRO RESPONSES

Experiments with rat lenses of similar ages to those used in *in vivo* experiments were performed in culture medium to determine how selenite acts on the lens. Isolation of lenses for *in vitro* studies predisposes the lens to additional stress due to handling and change of environment. Exposure of lenses to selenite *in vitro* also resulted in reductions in ATP content. Lens ATP was reduced 15% during a four-hour incubation in the presence of selenite, and continued to drop over a 24-hour period (Figure 3). A four-hour minimum exposure period was selected to allow for lens adaptation to culture conditions, and because metabolite concentrations in control lenses remain similar to those observed in *in vivo* control lenses. *In vitro* experiments with selenite impose a greater stress on the lens than observed *in vivo*. After 24 hours lenses were more fragile and selenite decreased lens ATP content by 80%.

The effect of selenite on glycolysis

In order to determine how selenite functions to decrease lens ATP content, the lens response to selenite was compared to treatment with marker inhibitors of metabolism. Copper ion has been reported to inhibit key glycolytic enzymes in rat brain postnuclear supernatants

(69) and in bovine lens (70). These responses may depend on the capacity of Cu^{+2} to interact with with protein thiol groups (71).

Since glycolysis is reported to be the primary ATP producing pathway in the bovine lens (19), reduction in lens ATP would be expected when lenses are incubated in the presence of copper. In these experiments, lens lactate production was used to estimate glycolytic activity. In 0.5mM $CuCl_2$, lens lactate production was inhibited 30% in vitro (Figure 4). However, 1.0mM selenite inhibited lactate production by only 10% in vitro (Figure 4). This result contrasts the elevated lens lactate production in response to selenite treatment in vivo (61). The difference in lactate production in vivo and in vitro may be explained by a potentially greater stress on the lens in vitro. This inhibition in lactate production may not be a direct effect of selenite on glycolysis, but could be explained by a loss of lens lactate dehydrogenase; these results are discussed below.

Results from experiments with combinations of inhibitors with Cu^{+2} revealed that selenite magnified the decrease in lens ATP concentration in the presence of $CuCl_2$. Therefore it was concluded that selenite may not act as an inhibitor of glycolysis. Azide, a respiratory inhibitor, and t-BHP, a general oxidant, also caused

further reductions in lens ATP in the presence of copper, although the interaction between copper and selenite resulted in more dramatic change. The synergistic effect by these agents indicated that ATP synthesis in the lens was not only affected by inhibiting glycolysis.

The effect of selenite on aerobic metabolism

Azide and cyanide interfer with electron transport by inhibiting cytochrome oxidase. These agents were used as indicators of mitochondrial metabolism. In the presence of azide and KCN lens lactate production was elevated, while lens ATP content was reduced (Figures 4, 6). These results indicated the expected metabolic regulation between ATP produced by the mitochondria, and that produced by glycolysis.

Experiments with two inhibitors were designed to explore further the site of selenite inhibition (Figure 6). Lens ATP was decreased the same extent by selenite alone, or in combination with KCN or azide. Apparently, selenite affects the mitochondria in a fashion similar to the typical respiratory inhibitors.

The glycerolphosphate cycle transfers cytosolic NADH to the mitochondria via sn-glycerol-3-phosphate (4). Lens glycerolphosphate increased 4-fold 24 hours after animals received a selenite injection (72). This

observation is consistant with impaired mitochondrial function by selenite. However, in vitro exposure of lenses to selenite for 4 hours decreased glycerolphosphate (61). This response is consistant with the decrease in lactate production by selenite in vitro and may reflect an increase in lactate dehydrogenase leakage in the presence of selenite.

Experiments to establish selenite affects on rat lens mitochondria were not possible due to limited quantities of tissue. Nor was it possible to obtain fuctional mitochondria using standard potocols for organelle isolation from bovine lens. Hence direct effects of selenite on rat liver mitochondria oxygen uptake were examined in supporting experiments (Table I). Selenite inhibited oxygen uptake with alpha-ketoglutarate, malate, and glutamate as substrates; yet with succinate as substrate oxygen uptake was virtually unaffected by selenite. Succinate reversed selenite-sensitive oxygen consumption to control levels. These data suggested selenite may function by inhibiting mitochondria at site I of electron transport.

Hightower and Harrison induced cataracts in cultured rabbit lenses with the ionophore, valinomycin by perturbing lens Ca^+, Na^+ and K^+ (73). The present data (figure 10) indicate valinomycin also decreased lens ATP.

Valinomycin decreases lens ATP by using the chemiosmotic potential maintained by respiration to pump cations, rather than to produce ATP (74). Copper ion magnified the decrease in ATP seen with valinomycin, which is consistent with an independent effect of these inhibitors on glycolysis and oxidative phosphorylation. It was surprising that selenite and azide had an additional effect on the reduction in ATP concentration observed with valinomycin. Perhaps the concentration of valinomycin (10^{-5}M) was not potent enough to totally prevent ATP synthesis in the intact tissue, therefore inhibition of electron transport by azide further depressed ATP synthesis. Additional experiments with higher concentrations of valinomycin were not completed.

Oxidative stress is another means by which lens metabolism may be compromised. As with selenite, t-BHP lowers lens lactate production and ATP content. However, selenite decreased ATP and lactate production more than t-BHP alone (Figure 5). This additional effect on ATP metabolism by selenite implied selenite affected lens energy metabolism by a mechanism other than oxidation. Treatment with t-BHP and valinomycin resulted in an synergistic inhibition of lens ATP content. Increased membrane permeability by t-BHP added to the already disturbed ion flux created by valinomycin. Therefore

more of the chemiosmotic potential would be required to control ion pumping in the mitochondria, and less would be available for ATP synthesis. These responses are consistant with a mitochondrial site of selenite action.

When relating selenite inhibition of lens ATP to the impact of azide or KCN in intact cells, another possibility must be considered. Exposing lenses to azide in vitro may lower lens ATP by indirectly inhibiting catalase. Lens H_2O_2 concentration was quantified to evaluate whether an accumulation of H_2O_2 could be a secondary result of treatment with azide or selenite. In both cases lens H_2O_2 concentration did not differ from controls (table II). Hence, the azide effect on lens ATP content resulted from a primary inhibition of mitochondrial function.

The effect of selenite on lens glutathione

Although selenite did not affect lens H_2O_2 (Table III), selenite decreased lens glutathione (Figure 11). Data (Figure 11) revealed glutathione content was similarly affected by t-BHP. In contrast, azide or valinomycin did not interfere with lens glutathione (Figure 11, 12, 13). Selenite may cause lens glutathione concentration to decrease by oxidizing glutathione to GSSG (43). Since GSSG is recycled by glutathione reductase, a loss of total glutathione is unexpected.

Shearer et al. reported that the decreased lens glutathione from selenite-induced cataracts was not accompanied by a stoichiometric increase in GSSG or protein-bound glutathione (75). They suggested glutathione was leaving the lens rather than accumulating as oxidation products.

A single IP injection of buthionine sulfoximine, a specific inhibitor of gamma-glutamylcysteine synthetase, decreased lens glutathione 90% in vivo (Figure 14). Under these conditions, buthionine sulfoximine did not affect lens ATP content (table II). It is significant that no cataract was observed in buthionine sulfoximine-treated rats even though glutathione concentration was 10% of controls. However, cataracts were observed in neonate mice which had received repeated doses of buthionine sulfoximine. In these mice, lens glutathione had decreased to undetectable levels.

Incubating lenses from buthionine sulfoximine-injected animals with selenite resulted in a loss of ATP content similar to the decrease in ATP caused by selenite alone. Buthionine sulfoximine had no effect on lens ATP content during a 4 hour incubation. Hence, ATP was not affected by buthionine sulfoximine in vivo or in vitro. Selenite, however, perturbed lens metabolism by both lowering lens ATP content and decreasing lens

glutathione concentration. It appears that the decrease in ATP may have greater implications for cataract formation than do decreases on glutathione content. Loss of both ATP and glutathione also precede cataract formation in galactose-induced cataract (76).

The release of lactate dehydrogenase from the lens

Lactate dehydrogenase leakage from cells is frequently used as an indicator of increased membrane permeability because it is more soluble than other marker enzymes, and because it affords easy detection (77). Cell trauma, with a subsequent increase in membrane permeability results in a release of lactate dehydrogenase (77). Lactate dehydrogenase detected in the incubation medium from the lens was used to indicate effects of inhibition on membrane permeability, and a means to clarify the different effects of selenite on lactate production observed *in vivo* or *in vitro*.

The release of lactate dehydrogenase from the lens of animals 24 hours after a selenite injection was not greater than control lenses. However, there was increased release of lactate dehydrogenase in the presence of selenite *in vitro* (Figure 15). The lower production of lactate after exposure to selenite could be explained because less lactate dehydrogenase is present in the lens. A greater effect on lens permeability was

observed in the presence of t-BHP since t-BHP may increase membrane permeability by causing lipid peroxidation. Rush, et al. reported 0.5mM t-BHP produces 70% elevation in lactate dehydrogenase leakage in isolated mouse hepatocytes (78). Together selenite and t-BHP caused a 27% increase in lens lactate dehydrogenase activity observed in the medium. The loss of lactate dehydrogenase from lenses seems small relative to the loss from isolated hepatocyte cells. The concentration of enzyme released by the lens reflects the effect of t-BHP on the peripheral epithelial layer relative to the other cells in the lens. The impact of these two agents could not be controlled by the lens glutathione peroxidase. Lens lactate dehydrogenase release was virtually unaffected by azide.

A disadvantage of using lactate dehydrogenase release as a measure of membrane permeability is the insult to which the lenses were subject may also inhibit lactate dehydrogenase activity (77). When lenses were treated with $CuCl_2$ the presence of lactate dehydrogenase could not be detected in the medium. Either lactate dehydrogenase activity was inhibited by $CuCl_2$, since copper has been reported as an inhibitor of hexokinase, pyruvate kinase and lactate dehydrogenase in rat brain extracts (69), or there may have been a direct effect of

Cu^{+2} on dye reduction, which is the basis for the lactate dehydrogenase assay.

In summary, in vitro experiments indicated selenite decreased lens ATP content by interfering with mitochondrial function (Table V), since no additional decrease in ATP occurred when selenite was combined with the respiratory inhibitor, azide. The combined treatment of lenses to t-BHP and Na_2SeO_3, however, resulted in an additive decrease in lens ATP, which is consistant with the general oxidative properties of t-BHP, rather than a specific effect on mitochondria. The increase in membrane permeability by t-BHP, as indicated by elevated LDH leakage, would also allow ATP and other metabolites to leak from the lens. Under the conditions of lens culture, these effects would be most apparent in the peripheral epithelial cell layer. Together Cu^{+2} and Na_2SeO_3 had a unique, synergistic effect on decreasing lens ATP concentration. This result may be attributed to the combined effect of selenite inhibiting mitochondria, the effect of Cu^{+} on glycolysis, and the effect of both Cu^{+} and selenite on decreasing lens thiols. The decrease in sulfhydryl would make the lens more suseptible to oxidation and therefore increase membrane permeability.

Table 5. SUMMARY OF INHIBITOR EFFECTS ON LENS METABOLISM IN VITRO

INHIBITOR	ATP	LACTATE PRODUCTION	TOTAL GLUTATHIONE	LDH[a] ACTIVITY
SeO_3^{-2}	15% ↓[b]	10% ↓	25% ↓	↑ 2x
KCN	20% ↓	36% ↑		
(+ SeO_3^{-2})[c]	--	--		
NaN_3	30% ↓	18% ↑	--	--
(+ SeO_3^{-2})	--	17% ↓		
(+ Cu^{+2})	55% ↓			
(+ t-BHP)	37% ↓			
Cu^{+2}	25% ↓	30% ↓	15% ↓	nd[d]
(+ SeO_3^{-2})	70% ↓	60% ↓		
(+ NaN_3)	51% ↓		--	
(+ t-BHP)	53% ↓		46% ↓	
Valinomycin	37% ↓		--	
(+ SeO_3^{-2})	50% ↓		31% ↓	
(+ NaN_3)	31% ↓		--	
(+ Cu^{+2})	71% ↓		31% ↓	
(+ t-BHP)	53% ↓			
t-BHP	14% ↓	14% ↓	25% ↓	↑ 3x
(+ SeO_3^{-2})	32% ↓	34% ↓		↑ 4x
(+ Cu^{+2})	55% ↓		44% ↓	
(+ NaN_3)	43% ↓		--	

[a] Lactate dehydrogenase activity in the incubation medium.
[b] Arrow indicates either inhibition (↓), stimulation (↑), or no effect (--).
[c] Parentheses designate simultaneous exposure to both inhibitors.
[d] Not possible to detect because of interference by Cu^{+2}.

The conclusion that selenite acts as a mitochondrial inhibitor is consistant with the *in vivo* studies in which the lens ATP decrease is accompanied by elevated lactate production. *In vivo* exposure of lenses to selenite was less traumatic, since a smaller dose was administered over a longer time interval. Altered membrane permeability was not detected in lenses from selenite injected animals (Figure 15). Hence it would be expected that normal regulation would result in an increased glycolytic flux in response to the decreased mitochondrial ATP production caused by selenite.

VI. CONCLUSIONS

1. Selenite decreased lens ATP concentration in rat lens primarily by action on mitochondrial function. ATP content was less in lenses from animals treated with selenite, while lactate production was elevated. Lens ATP concentration was decreased upon exposure to selenite *in vitro*. The interaction of selenite with typical inhibitors of energy metabolism lend additional support for mitochondrial inhibition by selenite.

2. Although glutathione concentration was decreased by selenite treatment *in vivo* and *in vitro*, experiments with BSO indicated a decrease in ATP is not required for lens glutathione to be reduced.

3. The differential response of lens lactate production to selenite *in vivo* and *in vitro* may be explained by an increased membrane permeability observed only *in vitro*. Hence, there is no need to consider selenite as an inhibitor of glycolysis.

4. The selenite-induced model for cataract is a relevant model for studying cataract formation. The model provides a rapid, reproducible means for investigating the biochemical changes in the pathogenesis of cataract.

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