LENS CALCIUM HOMEOSTASIS AND SELENITE CATARACT

By

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(ABSTRACT)

A 3- to 5-fold increase in Ca\(^{2+}\) accompanies cataract formation induced by selenite. The mechanism of selenite cataractogenesis involves calcium activation of calpain with subsequent proteolysis within the lens nucleus. This study was undertaken to investigate the biochemical mechanisms that lead to calcium accumulation in these circumstances. The components responsible for rat lens calcium regulation were defined by using either lens membrane vesicle preparations or intact lenses. Both Na\(^+\) gradient-dependent Ca\(^{2+}\) uptake and efflux occurred in lens membrane vesicles. Experiments with intact lenses showed that Na\(^+\)/Ca\(^{2+}\) exchange plays an important role in lens calcium regulation. ATP-dependent Ca\(^{2+}\) uptake and Ca\(^{2+}\)-dependent ATP hydrolytic activity have been characterized in lens membrane vesicles. Therefore, both Ca\(^{2+}\)-ATPase and Na\(^+\)/Ca\(^{2+}\) exchange participate in rat lens calcium regulation. Calcium accumulation in lenses treated by selenite may result from either increased influx (via non-selective cation channel), decreased efflux (via Ca\(^{2+}\)-ATPase and Na\(^+\)/Ca\(^{2+}\) exchange) or both. The selenite effects on the different components involved in lens calcium regulation were tested. Increased lens
calcium influx occurred in lenses from selenite-treated rats. The selenite effects on lens Ca\(^{2+}\)-ATPase activity were quantified with either ATP-dependent Ca\(^{2+}\) uptake or Ca\(^{2+}\)-dependent ATP hydrolysis. ATP-dependent Ca\(^{2+}\) uptake activity showed the greater extent of inhibition and, in contrast to ATP hydrolysis, was not reversed by the reducing agent dithiothreitol. Thus, possible uncoupling modification of lens Ca\(^{2+}\)-ATPase has occurred. Only a transitory decrease of Na\(^+\)/Ca\(^{2+}\) exchange activity was observed 48 h after injection of selenite. There was no inhibition of Na\(^+\),K\(^{+}\)-ATPase by selenite treatment. Mechanisms which might underlie selenite toxicity were studied. O\(_2\)^{•−}, possibly H\(_2\)O\(_2\), and unknown species were formed during GSH oxidation by selenite. Using a DNA strand breakage assay, the reactivity of these reactive species was determined. The reactive species generated by GSH and selenite caused DNA strand breaks. This DNA damage was protected by free radical scavengers. Due to the occurrence of O\(_2\)^{•−} and possibly H\(_2\)O\(_2\) during GSH reaction with selenite, O\(_2\)^{•−} or H\(_2\)O\(_2\) would be available to form cytotoxic hydroxyl radicals in the presence of iron or other transition metals. Therefore, an iron chelator, deferoxamine, was evaluated as a potential protective agent against the selenite-induced cataract in vivo. Deferoxamine protected against the posterior subcapsular cataract and delayed nuclear cataract induced by selenite, but higher doses of deferoxamine also provoked cataract at later time periods. In summary, selenite lens toxicity may be mediated by GSH oxidation, through which free radical species can be formed. These free radicals may cause damage of DNA, proteins and/or lipids. Particularly lens non-selective cation channels have increased permeability and Ca\(^{2+}\)-ATPase activity is less, and these together can lead to calcium accumulation in the lens.
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<table>
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<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>A-23187</td>
<td>Antibiotic A-23187; calcium ionophore</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DF</td>
<td>Deferoxamine</td>
</tr>
<tr>
<td>DMPO</td>
<td>5,5-dimethyl-1-pyrroline N-oxide</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-Dithiobis-(nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglybol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>Kd</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>MDA</td>
<td>Malonaldehyde</td>
</tr>
<tr>
<td>MPB</td>
<td>3-(N-maleidopropionyl)-biocytin</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium</td>
</tr>
<tr>
<td>NC</td>
<td>Nuclear cataract</td>
</tr>
<tr>
<td>NMDG</td>
<td>N-methyl-D-glucamine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>NSC</td>
<td>Non-selective cation channel</td>
</tr>
<tr>
<td>PCMBs</td>
<td>p-chloromercuribenzoate</td>
</tr>
<tr>
<td>PDA</td>
<td>Piperazine diacrylamide</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PI</td>
<td>Post-injection</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethysulfonyl fluoride</td>
</tr>
<tr>
<td>PSC</td>
<td>Posterior subcapsular cataract</td>
</tr>
<tr>
<td>Se</td>
<td>Selenite</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
</tbody>
</table>
INTRODUCTION

A single injection of sodium selenite into young rats leads to nuclear cataract formation within 72-96 h. The lens calcium concentration was increased 5-fold by 96 h after administration of selenite. This nuclear cataract arises as a result of activation of calcium-dependent neutral protease, calpain, which consequently degrades lens structural crystallins. Therefore, increased lens calcium plays a key role in nuclear cataract formation induced by selenite. In order to understand the mechanism of selenite cataractogenesis, we must investigate how selenite disturbs lens calcium homeostasis. This study was subdivided into three sections as follows:

1) Which components participate in lens calcium regulation?

2) Which components, responsible for lens calcium regulation, are affected by selenite treatment?

3) The potential mechanisms of selenite action.
LITERATURE REVIEW

The lens

Structure and function - The lens is a transparent biconvex body. The function of the lens is to focus light on the retina. In order to achieve this objective, the lens allows free passage of visible light. There are a number of unique characteristics that contribute to this property. The lens is avascular, thus eliminating absorption and scattering by components of blood which include the chromophore hemoglobin. The lens contains only a single layer of epithelial cells. Also, the lens contains a remarkably high concentration of proteins, ranging from 35 to 40% of the total lens weight. Most of these proteins are structural proteins called crystallins. Delaye and Tardieu have demonstrated that lens transparency is the result of short-range spatial order of these lens proteins (1).

The lens can be described as having three parts; the epithelial region, the cortex, and the nucleus (Figure 1). The lens consists of two cell types: epithelial cells and fiber cells. The epithelial cells differentiate into elongated fiber cells, losing their nuclei as well other cytoplasmic elements such as mitochondria and microsomes. Only the peripheral epithelial region of the lens remains active in metabolism. The lens is enclosed by a collagen glycoprotein elastic basement membrane, the capsule. This membrane acts more as a supporting wrapper than as a membrane barrier, since low molecular weight components such as sugars, amino acids, and lactate appear to pass through
Figure 1. Schematic diagram of a mammalian lens in cross section.
the capsule. The lens is supported by zonular fibers in a fluid and gel-like environment, on the anterior side by the rapidly replenished aqueous humor, and on the posterior side by the relatively static vitreous body. The dependency of the lens upon its environment is emphasized by the avascular nature of the tissue. All the nutrients must be obtained from, and all waste must be eliminated into, the surrounding fluids.

The fiber cells of the cortex and nucleus are metabolically inactive. Therefore, damaged intracellular macromolecules cannot be replaced (2-4). Thus, the inner region of the lens is dependent on the outer epithelial ring to maintain homeostatic control and to metabolize toxic compounds. This arrangement renders substantial portions of the lens particularly vulnerable to damage, since no apparent mechanism is available for repair (5).

**Metabolism** - The lens utilizes glucose as its source of metabolic energy. Since the O2-tension of the aqueous humor is low, most of its metabolism is anaerobic. The main pathway for the utilization of glucose is by way of Embden-Meyerhof or glycolytic reactions leading to lactic acid. The pentose phosphate pathway also contributes significantly to the breakdown of glucose to provide reducing equivalents (6).

Although adult fiber cells are incapable of protein synthesis, the lens contains several proteases, including neutral peptidase, endopeptidase, the ubiquitin ATP-dependent conjugation system, a 68-kD membrane-associated
protease and calpain (7-12). Two distinct forms of calpain have been characterized: calpain I, activated at low Ca$^{2+}$ (1-20 μM for half-maximal activity), and calpain II, requiring high Ca$^{2+}$ (250-750 μM for half-maximal activity) (13). Both calpains, as well as the capain inhibitor calpastatin, are found mainly in the epithelium and cortex of bovine lens (14,15). With purified calpain II from rat lens, several endogenous calpain substrates have been characterized; these include vimentin, intrinsic membrane proteins, and crystallins (16).

Some areas of the mammalian lens contain unusually high concentrations of glutathione. It is not evenly distributed. Its concentration is higher in the cortex than in the nucleus and highest in the epithelium. Glutathione in the lens is present primarily in the reduced state. Among its many important functions are: (1) maintenance of protein -SH groups in the reduced state; (2) participation in amino acid transport; (3) removal of xenobiotics by conjugation with hydrophobic compounds having an electrophilic center (a reaction catalyzed by glutathione S-transferase); and (4) detoxification of H$_2$O$_2$ by means of coupled reactions involving glutathione peroxidase, glutathione reductase and the hexose monophosphate shunt (17). Figure 2 illustrates the role of carbohydrate metabolism in affecting the redox state of glutathione.

Ascorbic acid (vitamin C) is present in the aqueous humor and the lens in high concentration in many species. There is accumulating evidence to show that ascorbic acid acts as both antioxidant and prooxidant. In a model system,
Figure 2. Scheme for reactions coupling the oxidation-reduction of glutathione and ascorbic acid with the hexose monophosphate shunt. Abbreviation used are: reduced glutathione (GSH); oxidized glutathione (GSSG).
ascorbic acid is able to scavenge various reactive oxygen species, but also can reduce transition metals that may in turn, produce free radicals through the Haber-Weiss reaction. Pirie has proposed that hydrogen peroxide might be formed in the aqueous humor during the oxidation of ascorbic acid (18), and this reaction was found to be catalyzed by light and riboflavin in vitro (Figure 2). Further study by Reddy et al. (19) showed that a deficiency of ascorbic acid in the diet of guinea pigs produced significant decreases of both H$_2$O$_2$ and ascorbic acid in the aqueous humor. This study also indicated a direct correlation between the concentrations of ascorbic acid and H$_2$O$_2$ in aqueous humor (19). Other in vitro studies with either γ-crystallin or bovine epithelial cells demonstrated a prooxidant effect of ascorbic acid (20,21). Ascorbate, along with riboflavin, inhibited $^{86}$Rb uptake in bovine lens epithelial cells in the presence of light. However, ascorbic acid was also shown to protect against lens damage induced by xanthine/xanthine oxidase free radical-generating system (22). Recently Devamanoharan et al. (23) demonstrated an anticataract effect of ascorbic acid in vivo.

**Lens proteins** - Of the total proteins in the rat lens, 86.5% (w/w) are lens-specific, water soluble proteins known as crystallins. They are separated into three categories, namely α-, β-, and γ-crystallins, and their physicochemical properties are listed in Table 1 (24). These different classes of crystallins can be readily separated by gel filtration chromatography and β-crystallin is resolved
<table>
<thead>
<tr>
<th>Properties</th>
<th>Class of crystallins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$</td>
</tr>
<tr>
<td>Electrophoretic mobility (towards the anode, at pH 8-9)</td>
<td>High</td>
</tr>
<tr>
<td>Range of isoelectric points shown by proteins</td>
<td>pH 4.8-5.0</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>Over $5 \times 10^5$</td>
</tr>
<tr>
<td>Molecular form</td>
<td>Polymers</td>
</tr>
<tr>
<td>Thiol content</td>
<td>Low</td>
</tr>
<tr>
<td>N-terminal</td>
<td>Masked</td>
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into two quite distinct components, $\beta_L$ and $\beta_H$. More recently, another class of crystallins, $\delta$-crystallin, have been identified exclusively in birds and reptiles (7). Delaye and Tardieu have shown that it is the short-range interactions between proteins that result in a system with minimal scatter (1). Further studies of interactions between crystallins will help us to understand the basis of lens transparency and may shed light on the molecular mechanism of cataract formation.

After extracting a lens homogenate with the usual buffer solutions (no urea and/or detergent), there is left a water-insoluble solution. Most of this insoluble fraction can be solubilized with hydrogen bond-breaking agents, such as 6 M urea, to yield the cytoskeleton proteins. The urea-insoluble fraction represents cell membrane proteins. The plasma membrane of lens fiber cells contains a protein with an apparent molecular weight of 26 kD (MP26), which comprises approximately one-half of the protein in this membrane system (25). A model of MP 26 based on amino acid sequences deduced from cDNA clones, showed the presence of six membrane-spanning hydrophobic $\alpha$-helices (characteristic of many intrinsic membrane proteins) and one amphiphilic transmembrane segment that could form an aqueous channel (26). With reconstituted bovine lens junction proteins (mainly MP26), voltage-dependent channels were formed (27). Hence, MP26 may form cell-to-cell channels in the lens.

Lens fiber cells contain many of the cytoskeletal elements present in
other non-muscle cells, as well as a lens-specific cytoskeletal component, beaded-chain filaments (28). The cytoskeleton of lens consists of three major components; microtubuli, intermediate filaments and microfilaments. It has been shown that the normal intermediate filament assembly is necessary to maintain the transparency of the lens (29).

**Lens ion regulation**

*Lens Na⁺ and K⁺ regulation* - In lens, as in other cellular tissues, the concentration of Na⁺ and K⁺ is 20 mM and 120 mM, respectively. Since the extracellular space of the lens is about 7 to 12% of the total lens mass, the Na⁺ and K⁺ are largely intracellular. In order to maintain a constant level of these ions, the lens must have a mechanism to regulate Na⁺ and K⁺ flux.

Following application of the patch clamp voltage technique to the apical membrane of a single lens epithelial cell, Jacob found single ion channel events similar to those normally associated with excitable tissues (30). The behavior of these channels suggested that they were sodium channels. Non-selective cation channels have also been described in membrane patches of the isolated epithelium of frog and human lenses (31-33). These channels allow both Na⁺ and K⁺ to pass, with a Na⁺:K⁺ selectivity ratio ranging from 3:1 to 1:1. However, the detailed mechanisms responsible for regulation of these channels have not been well elucidated. Harris and coworkers showed that the lens had the ability to actively pump cations (34). Palva and Palkama found that the
Na⁺, K⁺-ATPase is located in both epithelial and cortical regions (35). At least two different types of ouabain-sensitive ATPases have been demonstrated in the rabbit lenses (36). There is reasonable evidence to suggest that this Na⁺,K⁺-ATPase is electrogenic; that is, the current produced by the Na⁺,K⁺-ATPase is sufficient to hyperpolarize the lens resting voltage by about 10 mV (37). Therefore, Na⁺ probably enters the lens through Na⁺ specific or non-specific channel, and is pumped out by Na⁺,K⁺-ATPase.

**Lens calcium regulation** - The concentration of Ca²⁺ in the rabbit lens has been studied by McGahan *et al.* (38). Expressed in terms of lens-H₂O, it was 0.09 meq/L compared with 0.7 and 1.1 meq/L in aqueous and vitreous body, respectively. The concentration was less in the nucleus than in the whole lens. Only about 45% of the Ca²⁺ is readily exchangeable with ⁴⁵Ca²⁺, suggesting either slow diffusion or strong binding to cellular components. Hightower *et al.*, using a Ca²⁺-selective electrode to study the lenses of young rats, reported that total Ca²⁺ was 192 μM, and that free Ca²⁺ in cortex and nucleus was 0.2 and 0.5 μM, respectively (39).

Free Ca²⁺ is maintained in the cytoplasm of mammalian cells at a concentration that is 1000-fold lower than that of the extracellular milieu. This gradient is achieved by means of sophisticated homeostatic mechanisms. Given that the interior plasma membrane potential is -60 mV, an intracellular Ca²⁺ concentration of 0.1 - 0.2 M should be expected if Ca²⁺ were distributed
at electrochemical equilibrium (40). The first barrier to Ca\textsuperscript{2+} overflow into the cytoplasm is the natural impermeability of plasma membranes to ions. Most calcium passes the lens membrane through Ca\textsuperscript{2+} specific or non-specific channels. The existence of a "slow" calcium channel in the lens has been suggested, but not proven. In alloxan-diabetic rats, lens calcium increased from from 1.1 to 10.8 mM/Kg of dry wt, and 62% of the lenses became opaque. However, administration of the Ca\textsuperscript{2+}-channel blocker verapamil (25 mg/kg) reduced the occurrence of cataract to 3.9 percent, and lens calcium content remained low at 3.5 mM/Kg (42). In other experiments, a stretch-activated non-selective cation channel (NSC) has been shown in the frog lens with the clamp patch technique (31). The long term maintenance of steady and low intracellular Ca\textsuperscript{2+} depends on the operation of Ca\textsuperscript{2+}-extrusion mechanisms.

The Ca\textsuperscript{2+}-efflux mechanisms in the lens include both Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (41,43-49,51). The activity and distribution of Ca\textsuperscript{2+}-ATPase in the lens has been described by Iwata et al. and Borchman et al. (43,44,48). Borchman et al. showed that in rabbit and human lens, Ca\textsuperscript{2+}-ATPase activity was mainly located in the epithelium and cortex, but almost no activity was detected in the nucleus (44,48). A phosphorylated intermediate of Ca\textsuperscript{2+}-ATPase has been identified in the mammalian lens plasma membrane (45). Both ATP-dependent \textsuperscript{45}Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+}-dependent ATP hydrolysis were inhibited by calmodulin antagonists (46,47). The ATP-dependent \textsuperscript{45}Ca\textsuperscript{2+} uptake in lens membrane vesicles was stimulated by cAMP-dependent kinase
and inhibited by \( p \)-chloromercuribenzoate (47).

The existence of \( \text{Na}^+/\text{Ca}^{2+} \) exchange in the lens has been a point of controversy. Since \( \text{Ca}^{2+} \)-efflux in rabbit lenses was not affected by a change of \( \text{Na}^+ \)-gradient and incubation of rabbit lenses in \( \text{Na}^+ \)-free media did not influence \( \text{Ca}^{2+} \) accumulation, it appeared that \( \text{Na}^+/\text{Ca}^{2+} \) may be absent in lenses (38,49,50). Subsequently, Gaivan and Louis showed that calcium release from calcium loaded vesicles, prepared from calf lenses, was enhanced by \( \text{Na}^+ \), suggesting that the lens does contain \( \text{Na}^+/\text{Ca}^{2+} \) exchange capacity (47). By analysis of \( ^{45}\text{Ca}^{2+} \)-influx in the intact rat lens, Tomlinson et al. also supported the presence of \( \text{Na}^+/\text{Ca}^{2+} \) exchange in this species (51). The species difference may account for these conflicts. Perhaps rabbit lens has less need for a \( \text{Na}^+/\text{Ca}^{2+} \) exchange process due to a lower rate of calcium entry. Hadgkin and Keynes found that most of \( ^{45}\text{Ca}^{2+} \) which enters cells is subsequently bound (52), probably bind to negative charged phospholipids or proteins. Therefore, this constantly falling value of efflux rate due to isotope binding could also account for the negative results by Hightower et al. and others (38, 50).

Several \( \text{Ca}^{2+} \)-binding proteins have been isolated from the lens including calmodulin, EDTA-extractable proteins (EEP), \( \beta \)-crystallin and \( \delta \)-crystallin (53,54). The EEP contain calcium-binding sites with a total capacity of 25 mol/mol protein, with an apparent dissociation constant (Kd) of about 7.7 \( \mu \text{M} \). Equilibrium dialysis showed that four calcium atoms bind to one \( \delta \)-crystallin tetramer with an affinity of \( 4.3 \times 10^3 \text{ M}^{-1} \) (54). \( \beta \)-crystallin can bind four
calcium per aggregate unit of mass (160 KD), with an affinity of $2.6 \times 10^3 \text{ M}^{-1}$ (54). These different calcium binding proteins may serve as a buffering system in the lens.

**Cataract**

Cataract is defined as opacification that diminishes visual acuity of either the entire lens or localized sites therein. Cataract may occur as a senile change, as a result of trauma, as a result of metabolic or nutritional defects, as a result of toxicant substances, or as a consequence of radiation. A selected number of factors are listed in Table 2 (29). Senile cataract is most probably a multifactorial process.

**Oxidation and cataract** - Despite extensive research efforts in many laboratories and clinics, the specific and detailed mechanisms leading to human senile cataract have not yet been completely defined. Oxidation of membrane lipids and transport proteins, however, is an early and highly significant event in cataract formation (5). In advanced human cataract, there is an extensive oxidation of methionine and cysteine in lens proteins. With the oxidation of proteins, there is formation of HMW (high molecular weight) disulfide-linked aggregates (5). By scattering light these aggregates may contribute to the formation of opaque regions in the lens. Besides the formation of HMW aggregates, oxidative stress may also lead to the imbalance of lens ions which, in turn, may trigger an increase in proteolytic activity in the cataractous lenses.
### Table 2

**Some factors and conditions inducing cataract**

<table>
<thead>
<tr>
<th>Factors/Conditions</th>
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<tr>
<td>X-rays</td>
<td>Glucose</td>
<td>Chlorpromazine</td>
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<tr>
<td>β-rays</td>
<td>Galactose</td>
<td>Bleomycin</td>
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<tr>
<td>γ-rays</td>
<td>Xylose</td>
<td>Cyanate</td>
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<tr>
<td>Neutrons</td>
<td>Arabinose</td>
<td>Dimethyloxide</td>
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<tr>
<td>Protons</td>
<td>N-methy-N-nitroso urea</td>
<td>Inhibitors of choline</td>
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<td></td>
<td></td>
<td>esterase</td>
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<tr>
<td>UV</td>
<td>Iodoacetate</td>
<td>Inhibitors of cholesterol</td>
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<td></td>
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<td>synthesis</td>
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<tr>
<td>Infrared</td>
<td>Selenite</td>
<td>Oxidative stress; on</td>
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<td></td>
<td></td>
<td>amino acids, on lipids</td>
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<tr>
<td>Microwaves</td>
<td>Dopa</td>
<td>Diabetes</td>
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<tr>
<td>Ultrasound</td>
<td>Myleran</td>
<td>Aging</td>
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<td>Free radicals</td>
<td>Dinitrophenol</td>
<td>Genetic manipulation</td>
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<td>Naphthalene</td>
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H$_2$O$_2$ levels are increased in the aqueous humor of a significant number of cataractous patients (5). In vitro studies showed that H$_2$O$_2$ resulted in the uncoupling of the Na$^+$,K$^+$-pump (55). Na$^+$ is often elevated in the cataractous lens. An increase of human lens membrane permeability has been shown to correlate with age. Presumably this increase in Na$^+$ follows the increased ion traffic through the non-selective cation channels (56). Studies by Babizhayev et al. showed that there is accumulation of primary lipid peroxidation products (diene conjugates, cetodienes) in the initial stages of cataract, which may cause an increased membrane permeability of the lens membrane (57).

**Calcium and cataract** - In addition to the oxidation of proteins and peroxidation of lipids (5, 57), an increase in lens Ca$^{2+}$ was found in various types of both spontaneous and experimentally-induced cataracts (43). Recent studies highlight the role played by calcium in the development of senile cortical cataract (58, 59). Calcium toxicity to lens may include the following: (a) inhibition of Na$^+$,K$^+$-ATPase (60); (b) alteration of the permeability of membranes; (c) change of organization of membrane proteins in the gap junctions (61); acceleration of $\alpha$-crystallin aggregation to high molecular weight protein (62); (d) precipitation of calcium oxalate and phosphate; (e) Ca$^{2+}$-activated transglutaminase-mediated dimerization of $\beta$-crystallin (63); and (f) activation of proteolytic enzymes such as calpain leading to the degradation and aggregation of proteins (64). Lens calcium accumulation may occur either
from an increased influx, decreased efflux or both. External surface -SH groups of membrane proteins in lens are thought to be important in maintaining the normal impermeability of lens membrane to calcium (65, 66). Lenses damaged by oxidative stress may have both increased influx and decreased efflux of calcium due to the loss of critical membrane -SH groups. Recently Borchman et al. showed that the Ca\(^{2+}\)-ATPase activity can be inhibited by a low level of hydrogen peroxide in vitro (67). However, the mechanism which leads to the loss of calcium homeostasis in the cataractous lens has not yet been well defined.

**Selenite cataract** - A single subcutaneous injection of 30 nmol Na\(_2\)SeO\(_3\) per g body weight to preweanling rats results in the formation of posterior subcapsular cataract (PSC) 24 h post injection (PI), bilateral nuclear cataract between 72 h and 96 h, and cortical cataract between 15-30 days PI (68-71). The advantages offered by this model are convenience, rapidity, and reproducibility. Therefore, this model is useful for experimental studies on the biochemical mechanisms of cataract.

Total lens glutathione declines to about 40% of normal at 72 to 96 h after injection of sodium selenite, and its recovery is incomplete at 8 days PI (69). The depletion of GSH may be a result of non-enzymatic, catalytic oxidation of sulphydryl groups by selenite to form selenotrisulfides (72). The overall set of possible reactions in the lens is summarized in Figure 3. Despite
Figure 3. Non-enzymatic, catalytic oxidation of -SH groups by selenite. Abbreviations used are: reduced glutathione (GSH), Protein with free -SH group (P-SH); oxidized glutathione (GSSG); protein linked through disulfide bond (P-SS-P); selenodiglutathione (GS-Se-SG); protein linked through selenotrisulfide (P-S-Se-S-P).
the large decrease in lens glutathione after injection of selenite, no concurrent increase of oxidized glutathione (GSSG), protein mixed disulfide bond (protein-SSG), or protein crosslinking by disulfide bonds (PSSP) has been detected (69, 73). This has led Shearer et al. to suggest that selenite initiates cataract formation by oxidizing a small class of critical sulfhydryl groups (71). It seems that either selenite itself or selenite-induced stress is responsible for the oxidative damage. A recent study showed that superoxide can be generated when reduced glutathione reacts with selenite (74).

The formation of posterior subcapsular cataract at 12-30 h PI correlated with the appearance of medium to large vacuoles suggestive of localized hydration defects (71). The mechanism responsible for this kind of cataract is not clear. Histologically, the selenite-induced formation of cortical cataract appears to be related to at least two processes: a) suppression of mitosis and nuclear fragmentation leading to abnormal fibrogenesis, and (b) proteolysis of cortical fibers associated with extensive liquefaction of the cortex. It has been shown that selenite leads to a decrease of the number in elongated lens epithelial cells in vitro. It appears to be that both abnormal development of lens fiber cells and proteolysis are involved in the formation of cortical cataract. The mechanism leading to abnormal development of lens fiber cells probably is related to the DNA damage induced by selenite (75,76).

Selenite treatment may alter the activity of some enzymes. Lens catalase and superoxide dismutase were decreased 26% and 37%, respectively
following administration of multiple dose of selenite (70). ATP concentration was decreased and glutamine metabolism was suppressed in rat lenses after injection of selenite (77, 78). Selenite (100 μM) was shown to inhibit Na⁺,K⁺-ATPase activity by 25% in vitro, and the addition of glutathione reversed this inhibition (79). Diminished efflux of radiolabelled sodium in rabbit lens incubated with selenite has been observed by Hightower and McCready (80,81). These in vitro results suggest that the initiating event in selenite cataract formation could be inhibition of the Na⁺,K⁺-pump.

One of the most striking changes occurring during selenite cataract formation is a 3-5 fold increase in lens Ca²⁺ during the first 72 h PI (82). A consequence of this calcium elevation is activation of the proteolytic enzyme calpain II (83). Calpain is a non-lysosomal, calcium-activated, neutral protease found in many tissues including lens (14-16). Proteolysis of soluble and insoluble proteins in the nucleus occurs concurrent with the selenite opacity. Targets of calpain include α- and β-crystallins, and the MP26 and 66K membrane-associated polypeptides (71). The molecular mass of α- and β-crystallin fragments from selenite cataract is similar to those α- and β-crystallin fragments from lens incubated with calcium and to the proteolytic degradation products from lens proteins incubated with calpain II (83). These observations suggested that calcium plays an important role in the formation of cataract induced by selenite. Therefore, a proposed mechanism for selenite cataractogenesis involves calcium activation of calpain II with subsequent
proteolysis and irreversible damage to the lens nucleus (Figure 4). Partially proteolyzed \( \beta \)-crystallin has altered physical characteristics, leading to insolubility (71). This change may result in interactions with other crystallins to form aggregates and cause light scattering. Furthermore, the cysteine protease inhibitor, E64, has been shown to reduce the rate of cataract formation induced by selenite in rats (84). Since both activation of calpain and aggregation of proteins require an increase in cytoplasmic calcium, an investigation of calcium regulation is the key step to understand the mechanism of selenite cataract formation.

Several attempts have been made to determine the effect of selenite upon the components that participate in lens calcium regulation. Possible mechanisms for this increase in lens calcium in the selenite cataract include changes in \( \text{Na}^+ / \text{Ca}^{2+} \) exchange, inhibition of \( \text{Ca}^{2+} \)-ATPase and/or \( \text{Na}^+ . \text{K}^+ \)-ATPase, or opening of ion channels (Figure 4). The increase of calcium from selenite-treated rat lens may be a result of the oxidation of proteins and/or peroxidation of lipids. A deficiency of ATP might cause failure of \( \text{Ca}^{2+} \)-pump. Modification of lens proteins could alter \( \text{Ca}^{2+} \) binding affinity. Galvan and Louis studied calcium transport in plasma membrane vesicles from calf lens and reported that ATP-dependent \(^{45}\text{Ca}^{2+} \) uptake was inhibited 30% by exposure to 1 mM selenite (47). This concentration of selenite had no effect on passive permeability. Hightower and McCready found that an increase in lens \( \text{Na}^+ \) preceded changes in lens \( \text{Ca}^{2+} \) when rat lenses were incubated in 100 \( \mu \text{M} \)
Figure 4. Proposed components that may function in cataract formation induced by selenite.
\( \text{Na}_2\text{SeO}_3 \) for 24 h (80). Therefore, they suggested that osmotic stress led to calcium accumulation. They also reported a generalized increase in membrane permeability following selenite treatment. The concentration of selenite in these studies, however, was far above that which occurs in vivo. Moreover there is no general increase in lens sodium or hydration in vivo. Studies of lenses exposed to selenite in vitro may not be a satisfactory means of investigating the selenite cataract.

Some evidence suggests that lipid peroxidation occurs in selenite-induced cataract. Increased malonaldehyde (MDA) content in selenite-lenses was observed (70), and prevention of cataract formation followed by concurrent administration of the antioxidant, butylated hydroxytoluene (BHT) (85). Injection of ascorbic acid also protected against the cataract formation induced by selenite (23). Prior injection of the radioprotective compound WR-77913, which also has antioxidant properties, led to complete prevention of selenite cataract formation (86,87). Preliminary studies by Serhan et al. suggest that oxidized fatty acids can function as calcium ionophores (88). Whether or not this phenomenon is reproducible in the lens remains to be determined.

In summary, the selenite cataract offers a valuable model in which to study the functional state of an individual component which participates in the regulation of calcium homeostasis in the lens. Elucidation of the defects in calcium homeostasis in selenite-induced cataract may provide some clues for understanding the etiology of senile cataract.
EXPERIMENTAL DESIGN

All experiments were designed to address the following hypothesis:

*Selenite directly or indirectly acts to disrupt the normal mechanisms responsible for maintaining the normal calcium homeostasis. Selenite may do so through the oxidation of some critical membrane -SH groups that alters or impairs the function of membrane proteins.*

Therefore, the selenite effects on lens Ca\(^{2+}\)-influx, Ca\(^{2+}\)-ATPase, Na\(^+\),K\(^+\)-ATPase and Na\(^+\)/Ca\(^{2+}\) exchange were investigated, and the mechanisms that underlie these changes were explored.

Lens epithelial membrane protein thiol biot was used to localize these small oxidized -SH groups from selenite-treated rats. Experiments were also proposed to define reactive species formed during GSH oxidation by selenite. NBT, cytochrome c reduction and DNA strand breakage as a model system, the potential toxicity of these reactive species could be quantified. Finally, the effect of the iron chelator, deferoxamine, on selenite-induced cataract was quantified to test the involvement of the hydroxyl radical in the process of cataract formation.
EXPERIMENTAL PROCEDURES

Materials

Animals - Adult Sprague-Dawley rats were obtained from Dominion Laboratory (Dublin, VA). Litters were housed with their parents in individual cages through the weaning period, and were maintained at a relative humidity of 50-60%, 22±2°C, and a 12-h day/night cycle. Parent rats were provided laboratory chow and water ad libitum. All procedures involving animals conformed with the "Guide for the Care and Use of laboratory animals" (DHEW, NIH 86-23).

Chemicals and other supplies - Sodium selenite, Trolox C and 5,5-dimethyl-1-pyrroline N-oxide (DMPO) were obtained from Aldrich (Milwaukee, WI). 46CaCl2 (23.1 mCi/mg) was purchased from Du Pont (Wilmington, DE), and plasmid DNA (pMAMneo-CAT) was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). Glutathione reductase, catalase, bovine erythrocyte superoxide dismutase (SOD), dithiothreitol (DTT), calcium ionophore A-23187, ρ-chloromercuribenzenesulfonic acid (pCMBS), malachite Green, ammonium molybdate (NH₄)₆Mo₇O₂₄·4H₂O, MgATP, Na₂ATP, ouabain, histidine, sodium azide, mannitol, L-cysteine (HCl), L-ascorbic acid, phenylmethylsulfonyl fluoride (PMSF), benzamidine, leupeptin, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 3-(N-maleimidopropionyl)-biocytin (MPB), avidin-peroxidase (HRP), cytochrome c, nitro blue tetrazolium (NBT), bovine serum albumin (BSA), valinomycin, choline chloride, N-methyl D-glucamine, ethylenediaminetetraacetic acid disodium
(EDTA), and ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid (EGTA) were obtained from Sigma (St. Louis, MO). Hank's salts was obtained from KC Biological (Lenexa, KY). Filters (HAWP 0.45 μM) were purchased from Millipore (Bedford, MA), and the liquid scintillation counting solution, ECOLUME, was purchased from ICN (Irvine, CA). Utrex HNO₃, HCl and lanthanum oxide (1% w/v in 10% HCl) were obtained from J.T. Baker (Phillipsburg, NJ). Calcium standard (1000 ppm) and Coomassie Blue G-250 dye were obtained from Fisher Scientific (Pittsburgh, PA). Ampholyte, piperazine diacrylamide (PDA) and 4-chloro-1-naphthol were obtained from BioRad (Richmond, CA). All other chemicals were purchased from either Sigma or Fisher Scientific.

Methods

Induction of cataract - Nursing rats from 10- to 14-days old, were injected subcutaneously with a single dose of 20 mM sodium selenite in 0.9% NaCl in order to deliver 30 nmol selenite/g body wt. Control animals from the same litter were not injected with selenite (69).

Lens preparation - At the appropriate age or interval after injection of the animal with sodium selenite, the rats were killed by decapitation. Eyes were rapidly removed and opened by a posterior incision at the emergence of the optic nerve. Lenses were carefully extracted so as to avoid disturbance of the
capsule, washed briefly in Hank's TC 199 medium, blotted on moist, low-ash, filter paper, and transferred either to a suitable vessel for processing, or to a flask for incubation. A lens epithelial preparation, consisting of the epithelium and capsule, was removed from the lens by peeling the capsule, together with the adhering epithelial layer, from other parts of the lens. The cortex was separated from the dense nucleus by stirring the lens in 20 mM imidazole buffer (pH 7.5) for 5 min at 0-4°C; the remaining inner part of the lens was considered as the nucleus. If lens tissue was to be homogenized, the zonule was removed immediately. If an intact lens was used for incubation, the zonule remained attached until the incubation was completed.

*Calcium influx experiment* - In order to study lens calcium permeability changes after injection of the rat with selenite, the rat lens was cultured at 21°C in 300 mOsmolal medium pH 7.4, containing approximately 15 mM CaCl₂, 20 mM NaCl, 90 mM N-methyl-D-glucamine (NMDG), 5 mM KCl, 2 mM MgCl₂, 5 mM glucose and 10 mM HEPES buffer, adjusted pH to 7.4 (65). A temperature of 21°C was maintained to reduce active calcium extrusion. After 2 h, the zonule was removed from the lens, and the lens was then washed with 200 μl 0.5 mM EGTA. The lens was dried at 105°C for 48 h, weighed and then digested with 100 μl concentrated nitric acid at 200°C for 24 h (until nitric acid was dried), followed by 50 μl 30% (w/v) hydrogen peroxide at 200°C for overnight (until hydrogen peroxide was dried). This procedure was repeated for complete
digestion. The lens residue was dissolved in 1 ml 10% HCl, containing 1% lanthanum oxide. Total Ca\(^{2+}\) [µmol/g dry weight] was measured at 422.7 nm with a Perkin-Elmer Model 560 atomic absorption spectrophotometer, that was calibrated with a commercial calcium standard from 0-5 ppm (82).

**Effect of a Na\(^+\) gradient on the lens calcium regulation** - Lenses were incubated in two types of media, both at 300±5 mOsmolal, at either 21°C or 35°C, for time periods that varied depending upon the objective of the particular experiment. Modified Hank’s TC 199 medium, with either 20 mM NaCl [(-) transmembrane Na\(^+\) gradient] or 110 mM NaCl [(+) transmembrane Na\(^+\) gradient], was used in these experiments. For Ca\(^{2+}\) efflux experiments, lenses were first loaded with CaCl\(_2\) according to the protocol of the Ca\(^{2+}\) influx experiment, above. These "Ca\(^{2+}\)-loaded" lenses were transferred to modified Hank’s TC 199 medium for 4 h at 35°C. At different times of incubation, the lens was removed, washed, oven-dried, digested, dissolved in HCl/lanthanum, and total calcium was determined by atomic absorption spectrophotometer.

**Lens membrane vesicle preparation and characterization** - Lens plasma membrane vesicles were obtained by homogenizing approximately 6-10 lenses in 1 ml of 20 mM imidazole buffer (pH 7.5), and centrifuging at 7,000 x g for 20 min in a Beckman centrifuge (Model J21-B) at 4°C (47). The pellet was resuspended in 0.3-0.5 ml of 10 mM imidazole buffer (pH 7.5), containing 10%
sucrose, then rapidly frozen in liquid nitrogen and stored at -70°C. Sidedness of lens membrane vesicles was determined by the ouabain-sensitive Na⁺,K⁺-ATPase activity. Lens membrane Na⁺,K⁺-ATPase activity was determined in the presence or absence of 1 μM valinomycin, and total Na⁺,K⁺-ATPase activity was measured after 20 min pre-incubation of membranes in the presence of 0.1-0.2 mg sodium dodecyl sulfate (SDS)/mg protein at room temperature. The lens membrane vesicle preparations typically consisted of 38% unsealed vesicles, 44% right-side-out vesicles and 18% inside-out vesicles; this was in close agreement with the results of Galvan and Louis (47).

Na⁺ gradient-dependent Ca²⁺ uptake by vesicles - Lens membrane vesicles were quickly thawed from -70°C by incubating at 37°C, and centrifuged at 10,000 x g for 20 min (at 4°C). The vesicle pellet was resuspended (about 3 mg protein/ml) in 20 mM HEPES buffer, pH 7.4, containing 140 mM NaCl, and allowed to equilibrate at 0-4°C, for 4 h. Aliquots of the Na⁺-loaded vesicles were diluted 10-fold in the pH 7.4 HEPES buffer, containing either 140 mM NaCl or KCl, and 40 μM CaCl₂ (including 0.4 μCi ⁴⁰Ca²⁺ per 100μl). This suspension was incubated at 25°C and, at different time intervals, an aliquot was taken and filtered under vacuum through a Millipore filter (HAWP 0.45 μM). The filter was washed with 15 ml of ice-cold pH 7.4 buffer (120 mM KCl, 10 mM CoCl₂, 5 mM LaCl₃, 1 mM EGTA, and 20 mM HEPES buffer) (89,90). Ca²⁺ uptake was quantified using liquid scintillation solution at a
counting efficiency of 98%, in a LKB (model 1217 RACKBETA) Liquid Scintillation Counter.

*Na*⁺ gradient-dependent Ca²⁺ release from vesicles* - Lens membrane vesicles were loaded with Ca²⁺ in 20 mM HEPES buffer, pH 7.4, containing 140 mM KCl and 100 μM CaCl₂ (including 1.2 μCi ⁴⁵Ca²⁺/100 μl sample). This equilibration required a minimum of 6 h at 0-4°C. Ca²⁺ release was initiated by diluting the vesicles (3 mg protein/ml) 10-fold in 20 mM HEPES buffer (pH 7.4), 140 mM NaCl (↓⁺ Na⁺ transmembrane gradient) and 0.1 mM EGTA at 25°C. In control experiments, 140 mM KCl (↑⁻ Na⁺ transmembrane gradient) was substituted for the NaCl. At different time intervals during the vesicle incubation, an aliquot of membrane vesicles was removed and filtered. The filter was washed, and the Ca²⁺ uptake was determined by scintillation counting, as previously described.

*Inorganic phosphate assay* - This assay is based on the interaction of the phosphomolybdate complex with malachite green to form a colored complex (91). The malachite reagent was prepared by mixing 3 parts of 0.045% malachite green (oxalate salt) in deionized distilled H₂O with 1 part 4.2% ammonium molybdate solution (in 4 M HCl), then stirring for 30 min at room temperature. The malachite reagent was filtered, and 0.1 ml Triton X-100 was added per 5 ml of the malachite solution. Due to continuous hydrolysis of ATP
and ADP in an acidic solution, which in turn interferes with the inorganic phosphate assay, citrate was added, along with the malachite reagent to each sample, so that the color reaction was rendered insensitive to nascent phosphate. At room temperature 1.725 ml malachite green reagent were added to a 75 µl sample, and the solution was mixed. After 1 min, 200 µl 34% sodium citrate (w/v) solution were added, and the solution was mixed again. The absorbance of the resulting colored solution was read at 645 nm in a LKB UITROSPEC II uv/vis spectrophotometer within 30 min (91).

**Lens Ca$^{2+}$-ATPase assay** - Two lenses were combined together and homogenized with a glass homogenizer in 0.5 ml 20 mM imidazole buffer, pH 7.5, and this homogenate was used directly in the assay. In the reaction tube, 40 µl of homogenate were added to 445 µl of either Buffer A (100 mM KCl, 50 mM HEPES, 5 mM MgCl$_2$, 1 mM EGTA, pH 7.4) or Buffer B (Buffer A plus 1.1 mM CaCl$_2$); 25 µl of 20 mM MgATP were added to start the reaction. For lens membrane preparations, 25 µl were used for each assay. Samples were incubated at 37°C for 60 min, and the reaction was stopped with the addition of 50 µl ice-cold 6 N HCl. After centrifugation at 10,000 rpm for 5 min (in a microcentrifuge at 4°C), the phosphate released was analyzed using the inorganic phosphate assay method. Ca$^{2+}$-ATPase activity was defined by subtracting the inorganic phosphate measured in the presence of EGTA alone, from that inorganic phosphate obtained in the presence of calcium; this was
reported as nmol Pi/lens/h.

**ATP-dependent $^{45}$Ca$^{2+}$ uptake in lens membrane vesicles** - ATP-dependent Ca$^{2+}$-uptake was initiated by the addition of 20 μl vesicles (approximately 60 μg protein) to 180 μl Ca$^{2+}$-uptake medium (120 mM KCl, 20 mM HEPES (pH 7.4), 2 mM MgCl$_2$, 120 μM CaCl$_2$, including 0.5 μCi $^{45}$Ca$^{2+}$/100 μl, with or without 2 mM MgATP. Uptake of $^{45}$Ca$^{2+}$ was determined after incubation of the vesicles at 37°C for 5 min, then filtering 100 μl of the suspension through a 0.45 μM filter (47). The filter was washed with 15 ml ice-cold medium (120 mM KCl, 10 mM CoCl$_2$, 5 mM LaCl$_3$, 0.5 mM EGTA and 20 mM HEPES, pH 7.4) (89,90). Calcium remaining on the filter, assumed to be internalized calcium, was quantified by liquid scintillation counting. The $^{45}$Ca$^{2+}$ pumping activity was defined by subtracting the Ca$^{2+}$-uptake measured in the absence of ATP from that obtained in the presence of ATP.

**Na$^+$,K$^+$-ATPase assay** - Na$^+$,K$^+$-ATPase activity was determined according Galvan and Louis (47) with the following modifications. A pair of lenses was homogenized with a glass homogenizer in 0.5 ml 20 mM imidazole buffer (pH 7.5). The Na$^+$,K$^+$-ATPase activity in the lens homogenate (40 μl) was assayed at 37°C in a medium (1 ml) which contained 140 mM NaCl, 20 mM KCl, 3 mM MgCl$_2$, 30 mM histidine (pH 7.5), 0.2 mM EGTA and 2 mM ATP, with or without 1 mM ouabain. The Na$^+$,K$^+$-ATPase activity was defined as the
activity which is inhibited by 1 mM ouabain. Inorganic phosphate in the samples was determined by the malachite green method (91).

_Lens membrane protein thiol blot_ - Epithelia were isolated and homogenized with a 0.2 ml micro tissue grinder in 40 mM Tris-Cl, pH 7.4, containing 2.5 mM EGTA, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 2.5 μM leupeptin (approximately 12 epithelia/600 μl). The buffer was bubbled with argon for 10-20 min to prevent -SH oxidation prior to homogenization. All of these procedures were performed at 4°C. The homogenate was centrifuged at 10,000 rpm for 15 min (in a microcentrifuge at 4°C). The pellet was homogenized again, and resuspended in 0.75 ml buffer containing 0.3 mg/ml 3-(N-maleimidopropionyl)-biocytin (MPB), and this suspension was stirred for 1 h at room temperature (92). The sample was then centrifuged, and the pellet was dissolved in urea solubilization buffer (5.4 g urea, 0.4 ml NP-40, 1 ml 20% (w/v) ampholyte (pH 3-10), 0.2 ml 2-mercaptoethanol, added H₂O to 10 ml, adjust pH with NaOH to 9.5) for electrophoresis. Two-dimensional gel electrophoresis was performed according to the protocol described (93). A mini-gel apparatus (Hoefer Scientific Instruments, Model 250) was used. Different ampholytes were mixed using the ratio of 1:1:2 (pH 4-6:pH 5-8:pH 3-10). PDA was used instead of bis-acrylamide in the isoelectric focusing, and the second dimensional gel. About 15 μg protein was loaded and electrophoresis was carried out at 700 V for 4-5
h at 4°C. Electrophoresis was in 20 mM NaOH as upper tank solution and 8.7 mM phosphoric acid as lower tank solution. The second dimension was carried out on a 8% SDS acrylamide gel. This gel was stained using the silver stain method. The transfer of the gel to nitrocellulose membrane was carried out with Bio-Rad semi-dry transfer cell at 15 volts for 30 min at room temperature. The transfer buffer contained 48 mM unbuffered Tris, 39 mM glycine, 0.13 mM SDS and 20% methanol. After transfer, the nitrocellulose filter was placed in blocking buffer (1% non-fat milk in phosphate buffer saline (PBS, 137 mM NaCl, 2.68 mM KCl, 4.29 mM Na₂HPO₄·7 H₂O, and 1.47 mM KH₂PO₄) overnight at 4°C, then probed for transferred proteins with avidin-peroxidase. 4-Chloro-1-naphthol was used as the peroxidase substrate (92). This whole procedure can be summarized with the following scheme.

\[
\text{SH} \\
\quad \downarrow \text{MPB treatment} \\
\quad S \rightarrow \text{MPB} \\
\quad \downarrow \text{Two dimensional gel} \\
\quad \downarrow \text{Blot to nitrocellulose} \\
\quad \downarrow \text{Avidin-HRP} \\
\quad S \rightarrow \text{MPB} \rightarrow \text{HRP} \\
\]

Scheme for thiol (-SH) blot

Cytochrome c or NBT reduction by GSH reaction with selenite - The reaction
mixture (2 ml), consisted of either 10 μM cytochrome c, or 100 μM Nitro blue tetrazolium (NBT), 50 mM potassium phosphate buffer, pH 7.4, with EDTA (0.1-10 mM), and different concentrations of reduced glutathione and sodium selenite. The rate of cytochrome c reduction was monitored at 550 nm, and the rate of NBT reduction was measured at 560 nm spectrophotometrically (94). The extent of the reduction of cytochrome c or NBT, in the absence or presence of superoxide dismutase, was used to assess the possible production of the superoxide anion. The reduction of either cytochrome c or NBT was also measured under anaerobic conditions by bubbling the sealed cuvette with argon for 30 min prior to starting the reaction.

**Oxygen uptake** - Oxygen uptake was measured at 30°C using a Clark oxygen electrode (Gilson Medical Electronics). The reaction mixture was in a volume of 1.5 ml containing different concentrations of GSH and selenite. The results were expressed as nmol/ml of oxygen consumed per unit time (95).

**Determination of glutathione** - Total glutathione was determined by a coupled enzyme assay (96), and reduced glutathione was determined by a direct DTNB assay (97). For the coupled assay, lenses were weighed and extracted with 0.9 M perchloric acid/0.05 M phosphoric acid (0.5 ml/lens) by homogenizing at room temperature. Total glutathione was determined in a 1.2-ml reaction mixture containing 400 μl Reagent I (110 mM Na₂HPO₄·7H₂O, 40 mM
NaH₂PO₄·H₂O, 15 mM EDTA, 0.04% BSA, 0.3 mM DTNB (pH 6.9), 320 μl Reagent II (1 mM EDTA, 0.02% BSA, 50 mM imidazole buffer (pH 7.12)), 1.5 units of glutathione reductase, and 400 μl of diluted (40 fold) acid-extracted sample. The reaction was started by the addition of 80 μl of 9.0 mM NADPH. The change of absorbance was recorded for 2 min at 412 nm using a LKB Ultraspec II (Model 4050 uv/vis) spectrophotometer. Oxidized glutathione (GSSG) was used as standard.

For some in vitro experiments, the reduced glutathione was determined as follows: an aliquot of sample was diluted 20-fold in 0.1 M Tris-Cl buffer (pH 8.1), then 50 μl 10 mM DTNB methanol solution was added into 2 ml of sample (97). After 5 min the absorbance at 412 nm was measured, and reduced glutathione concentration of the sample was determined by using reduced GSH as standard.

**Assay for DNA strand breaks** - DNA cleavage was measured by the conversion of closed circular supercoiled plasmid DNA to the nicked form (98). Briefly, 0.26 μg plasmid (pMAMneo-CAT) DNA was incubated for 30 min at room temperature with 20 μl 50 mM potassium phosphate buffer (pH 7.4) containing 100 μM EDTA, 1 mM GSH, and 25 μM sodium selenite. The DNA was electrophoresed on 0.8% agarose gel in 40 mM Tris-acetate, 2 mM EDTA buffer, pH 8.0. The gel was stained with ethidium bromide and photographed with Polaroid 665 film. The corresponding negatives were scanned using a
Shimadzu Model CS-9000 scanning densitometer (Kyoto, Japan).

**Protein assay** - Protein determination was routinely performed using the Bradford Coomassie Blue protein assay (99). The stock dye solution was prepared as follows: 100 mg Coomassie Blue G-250 dye were dissolved in 50 ml 95% ethanol, 100 ml 85% (w/v) H₃PO₄ were added, and the final volume was adjusted to 200 ml with deionized distilled H₂O. The stock Coomassie Blue dye solution was diluted 5-fold, and 5 ml of the dilute dye solution were added to each sample. The protein-dye sample absorbance was measured at 595 nm in a uv-vis spectrophotometer. Bovine serum albumin (0-80 μg) samples prepared in the same medium as the unknown samples, were used to generate a protein standard curve.

**Ion analysis** - Control and selenite-treated rats were decapitated at different times after injection; lenses were removed with capsules intact, quickly washed (50 μl 0.5 mM EGTA/lens), and blotted on filter paper. Lenses were dried, and two lenses were digested by concentrated nitric acid and 30% H₂O₂ as previously described. The residue was dissolved in 10% HCl for sample analysis. Simultaneous measurements of Ca²⁺, K⁺, Na⁺ were accomplished with inductively coupled plasma (ICP) analysis using the Jarrel-Ash ICAP 9000 instrument (Franklin, MA) in the Soil Testing and Plant Analysis Laboratory, VPI&SU, Blacksburg, VA. Alternately, Ca²⁺ was analyzed using the same
digest, but dissolving the salts in 0.1% lanthanum oxide (spectral releasing agent) in 10% HCl, and employing atomic absorption spectrophotometer (82).

**Statistical methods** - All data are expressed as mean ± S.E. Analytical data for ion content were evaluated over time, using standard linear least squares or exponential regression analysis on means of values from lens of the same rat litter. Student’s t-test was used to compare means of values from control and selenite-treated animals; a difference was considered to be significant when P < 0.05.
RESULTS

1. Components participating in lens calcium regulation

1.1. Na\(^+\) gradient-dependent Ca\(^{2+}\) uptake and release from membrane vesicles - To investigate the occurrence of Na\(^+\)/Ca\(^{2+}\) exchange in lens membrane vesicles, \(^{45}\)Ca\(^{2+}\) uptake was determined both with and without an outwardly-oriented Na\(^+\) gradient at 25°C. Over 90% of Na\(^+\) gradient-dependent Ca\(^{2+}\) uptake occurred during the first 5 min of incubation (Figure 5, Table 3). The maximal \(^{45}\)Ca\(^{2+}\) uptake at 10 min was 2.51 ± 0.15 nmol/mg protein in the presence of a transmembrane Na\(^+\) gradient, in contrast to 0.98 ± 0.06 nmol/mg protein without transmembrane Na\(^+\) gradient. Accumulated Ca\(^{2+}\) was rapidly and completely released by treatment of vesicles with the Ca\(^{2+}\) ionophore, A 23187, indicating that Ca\(^{2+}\) was within the intravesicular space. The reversibility of Na\(^+\)/Ca\(^{2+}\) exchange was also quantified; that is, Na\(^+\) gradient-dependent Ca\(^{2+}\) efflux from lens membrane vesicles, preloaded with calcium, was demonstrated in the presence or absence of an inwardly-oriented Na\(^+\) gradient. There was a Na\(^+\)-gradient Ca\(^{2+}\) efflux from lens membrane vesicles preloaded with \(^{45}\)Ca\(^{2+}\) (Figure 6, Table 4). These data provide the compelling evidence of the occurrence of Na\(^+\)-Ca\(^{2+}\) exchange in rat lens.

1.2. Na\(^+\) gradient effect on Ca\(^{2+}\) content in rat lens - To determine the contribution of Na\(^+\)/Ca\(^{2+}\) exchange to Ca\(^{2+}\) homeostasis in the lens, lenses from 15-day old rats were incubated in medium containing either normal (110
Figure 5. Effect of Na\textsuperscript{+} gradient on Ca\textsuperscript{2+} uptake by lens membrane vesicles. Time-dependent \textsuperscript{45}Ca\textsuperscript{2+} uptake in the presence (●⋯⋯●) or absence (■——■) of an outwardly-oriented Na\textsuperscript{+} gradient at 25°C. One µM Ca\textsuperscript{2+} ionophore, A 23187, was added after 10 min (arrow). This figure was derived from the data of table 3.
Table 3

Effect of Na\(^+\) gradient on Ca\(^{2+}\) uptake by lens membrane vesicles

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Ca(^{2+}) uptake, nmole</th>
<th>(+) Gradient</th>
<th># Sample</th>
<th>(-) Gradient</th>
<th># Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1.73 ± 0.19</td>
<td>3</td>
<td>0.75 ± 0.08</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.33 ± 0.17</td>
<td>4</td>
<td>0.84 ± 0.02</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.39 ± 0.16</td>
<td>7</td>
<td>0.85 ± 0.046</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>2.56 ± 0.18</td>
<td>5</td>
<td>0.89 ± 0.03</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.52 ± 0.16</td>
<td>6</td>
<td>0.98 ± 0.058</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6. Effect of Na\(^+\) gradient on Ca\(^{2+}\) efflux from lens membrane vesicles. Time-dependent \(^{45}\)Ca efflux in the presence (●·····●) or absence (■——■) of an inwardly-oriented Na\(^+\) gradient at 25°C. This figure was derived from the data in table 4.
### Table 4

*Effect of Na\(^+\) gradient on Ca\(^{2+}\) efflux from lens membrane vesicles*

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Ca(^{2+}), percent remaining in vesicles</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-) Gradient</td>
<td>n</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>70.1 ± 10.1</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>76.0 ± 8.80</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>72.3 ± 4.10</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>68.1 ± 8.80</td>
<td>3</td>
</tr>
<tr>
<td>15</td>
<td>55.7 ± 5.50</td>
<td>3</td>
</tr>
<tr>
<td>20</td>
<td>77.7</td>
<td>1</td>
</tr>
</tbody>
</table>
mM) or reduced Na⁺ content (20 mM) at 35°C. In the latter case, either choline or N-methyl-D-glucamine (NMDG) was used as the cation substitute in order to maintain constant ionic strength and membrane potential. After 8-h incubation, the lens calcium content was determined by atomic absorption spectrophotometer. The results of representative experiments are shown in Table 5. In the absence of transmembrane Na⁺ gradient, the rat lens cannot maintain a normal, low level Ca²⁺.

1.3. Na⁺ gradient effect on Ca²⁺ efflux in lenses preloaded with Ca²⁺ - Lenses were loaded with calcium in medium containing 15 mM CaCl₂ at 21°C for 2 h. Then these lenses preloaded with Ca²⁺ were incubated at 35°C for 4 h in the modified Hank’s TC 199 medium either with or without a transmembrane Na⁺ gradient. Only the lens, incubated with transmembrane Na⁺ gradient, returned its calcium content to near basal level (Table 6).

During the incubation periods used in these experiments there was no swelling of intact lens as determined from lens weight. Lens transparency was cloudy in lenses with >0.8 μmol Ca²⁺/g dry wt, and lenses loaded with Ca²⁺ were opaque. The Ca²⁺-loaded lenses, incubated in the medium containing a transmembrane Na⁺ gradient, had improved transparency after incubation.

1.4. ATP-dependent ⁴⁵Ca²⁺ uptake in lens membrane vesicles - A time course of ATP-driven Ca²⁺ uptake in rat lens plasma vesicles is shown in Figure 7.
Table 5

**Na\(^+\) gradient effect on the Ca\(^{2+}\) concentration in rat lens**

Lenses were incubated in modified Hank's TC 199 medium at 35°C for 8 h with transmembrane Na\(^+\) gradient or without transmembrane Na\(^+\) gradient. The lens calcium content was determined by atomic absorption spectrophotometer. Mean values ± SE are reported.

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>Ca(^{2+}) content (μmol/g dry wt)</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>110 mM NaCl</td>
<td>0.53 ± 0.05</td>
<td>8</td>
</tr>
<tr>
<td>20 mM NaCl, 90 mM NMDG</td>
<td>0.84 ± 0.05*</td>
<td>7</td>
</tr>
<tr>
<td>20 mM NaCl, 90 mM Choline</td>
<td>0.66 ± 0.05</td>
<td>7</td>
</tr>
</tbody>
</table>

* Significantly greater than control lens (110 mM NaCl), p < 0.01.
Table 6

**Na**\(^+\) gradient effect on the **Ca**\(^{2+}\) efflux in

**Ca**\(^{2+}\)-loaded rat lenses

Lenses from 15-day old rats were loaded with Ca\(^{2+}\), then transferred to modified Hank's TC199 medium containing either 110 mM NaCl [(+)-gradient or 20 mM NaCl[(-)-gradient] for an additional 4-h incubation at 35°C. The lens calcium content was determined by atomic absorption spectrophotometer. Mean values ± SE are reported.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ca(^{2+}) content (µmol/g dry wt)</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+})-loaded</td>
<td>3.78 ± 0.58</td>
<td>7</td>
</tr>
<tr>
<td>110 mM NaCl</td>
<td>0.81 ± 0.08</td>
<td>11</td>
</tr>
<tr>
<td>20 mM NaCl, 90 mM NMDG</td>
<td>2.55 ± 0.21*</td>
<td>11</td>
</tr>
<tr>
<td>20 mM NaCl, 90 mM Choline</td>
<td>1.99 ± 0.16*</td>
<td>4</td>
</tr>
</tbody>
</table>

* significantly greater than control lenses (110 mM NaCl), p<0.01.
Figure 7. Time course of ATP-dependent Ca$^{2+}$ uptake. Lens membrane vesicles were used to take up $^{45}$Ca$^{2+}$ in the presence (■——■) or absence (●——●) of 2 mM MgATP as described under the experimental procedures. The calcium concentration for these experiments was 100 μM.
The reaction was carried out in a K⁺-rich solution to avoid interference by Ca²⁺ uptake due to Na⁺/Ca²⁺ exchange. In the presence of 2 mM MgATP, there is a rapid accumulation of ⁴⁵Ca²⁺ during the initial 15 min of incubation (Figure 7). The calcium concentration effect on ⁴⁵Ca²⁺ transport activity is shown in Figure 8. Maximum Ca²⁺ uptake by lens membrane vesicles occurred in 120 μM Ca²⁺.

1.5. Ca²⁺-ATPase and Its Regional Distribution in the Lens - Ca²⁺-ATPase activity was detected in lens homogenates, as well as in lens membrane vesicle preparations. Ca²⁺-ATPase activity was defined as the difference in inorganic phosphate release in the presence and absence of free calcium. Total Ca²⁺, Mg²⁺ dependent ATPase activity from lens homogenates was 294 ± 14.7 nmol/lens/h, and the Ca²⁺-ATPase activity from the homogenates was 37.8 ± 3.6 nmol/lens/h. Only 18.4 ± 3.9 nmol/lens/h of Ca²⁺-ATPase activity was observed in lens membrane vesicle preparations. Exogenous calmodulin failed to stimulate the Ca²⁺-ATPase activity of lens membrane vesicle preparations. The distribution of Ca²⁺-ATPase activity in the lens was studied with membrane vesicle preparations. About one-third of the Ca²⁺-ATPase activity is distributed in the epithelial region (6.6 ± 2.0 nmol/lens/h, n = 6), and the remainder is located in the cortical region (10.4 ± 1.55 nmol/lens/h, n = 5) (Figure 9). No activity was detected in the nucleus.
Figure 8. Ca$^{2+}$ concentration dependence of ATP-dependent Ca$^{2+}$ uptake. Ca$^{2+}$ pump activities were measured in the presence of various concentrations of Ca$^{2+}$ as described in Materials and Methods. The amount of ATP-dependent $^{45}$Ca$^{2+}$ uptake in 5 min was taken as the initial rate of the Ca$^{2+}$ pump.
Figure 9. Ca\textsuperscript{2+}-ATPase distribution in rat lens. Lens membrane vesicles prepared from different regions were used for the Ca\textsuperscript{2+}-dependent ATP hydrolysis assay. No Ca\textsuperscript{2+}-ATPase activity was detected in the nuclear region. The mean values from at least 3 preparations were reported.
2. Which components, responsible for lens calcium regulation, are affected by selenite treatment?

2.1. Selenite effect on $\text{Ca}^{2+}$ influx in the rat lens - In these experiments, age-matched control lenses and lenses from selenite-treated animals were incubated at $21^\circ\text{C}$ for 2 h in medium containing high CaCl$_2$ and in the absence of a transmembrane Na$^+$ gradient. Then lens calcium content was analyzed by atomic absorption spectroscopy. Calcium accumulation (the final calcium concentration in the lens after incubation, minus the endogenous calcium prior to the incubation) in rat lenses at different times after administration of selenite, is shown in Figure 10 and Table 7.

These results demonstrated an increased calcium influx in the lenses from selenite-treated rats. Influx rates almost doubled at 36 h after injection of selenite, but returned to the control rate at 72 h PI. In order to investigate the possible role of membrane -SH groups in maintenance of membrane resistance to calcium passage, an impermeable -SH modifying reagent, $\rho$CMBS, was added to control lenses. Increased calcium influx occurred in the lenses treated with $\rho$CMBS (Table 8), suggesting that the increased calcium influx in lenses from selenite-treated animals might have been related to membrane -SH modification. Dithiothreitol (DTT), a thiol reductant, had no effect on $\text{Ca}^{2+}$ influx of control lenses, but reversed the increased calcium influx in the lenses from selenite-treated rats (Table 9). These results showed that the rat lens calcium permeability was increased by selenite treatment, and this increased
Figure 10. Ca$^{2+}$-influx in rat lens. Lenses from age-matched control (■—■) or selenite-treated rats (○——○) were used for that assay. Total Ca$^{2+}$ concentration was determined by atomic absorption spectrophotometry. Each data point represents the means of at least 3 samples.
Table 7

**Effect of selenite on Ca\(^{2+}\)-influx**

Lenses were incubated at 21°C for two h in medium containing 15 mM CaCl\(_2\) in the absence of a Na\(^+\) gradient. Lens calcium content was determined by atomic absorption spectrophotometer. Means ± SE are reported.

<table>
<thead>
<tr>
<th>Time after Treatment</th>
<th>Ca(^{2+}) (μmol/g dry weight)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final n</td>
<td>Initial n</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h Pl</td>
<td>3.95 ± 0.44 10</td>
<td>0.63 ± 0.08 7</td>
</tr>
<tr>
<td>36 h Pl</td>
<td>4.10 ± 0.27 6</td>
<td>0.76 ± 0.07 3</td>
</tr>
<tr>
<td>48 h Pl</td>
<td>4.07 ± 0.38 9</td>
<td>0.61 ± 0.03 7</td>
</tr>
<tr>
<td>72 h Pl</td>
<td>4.14 ± 0.67 8</td>
<td>0.75 ± 0.06 9</td>
</tr>
<tr>
<td>96 h Pl</td>
<td>3.20 ± 0.51 12</td>
<td>0.69 ± 0.06 7</td>
</tr>
<tr>
<td>Selenite-treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h Pl</td>
<td>4.39 ± 0.26 12</td>
<td>0.80 ± 0.03 3</td>
</tr>
<tr>
<td>36 h Pl</td>
<td>8.76 ± 1.31 6</td>
<td>1.12 ± 0.03 3</td>
</tr>
<tr>
<td>48 h Pl</td>
<td>7.03 ± 0.33 10</td>
<td>1.25 ± 0.09 9</td>
</tr>
<tr>
<td>72 h Pl</td>
<td>5.69 ± 0.61 5</td>
<td>2.54 ± 0.29 10</td>
</tr>
<tr>
<td>96 h Pl</td>
<td>6.60 ± 0.41 11</td>
<td>3.38 ± 0.44 4</td>
</tr>
</tbody>
</table>

* p<0.01 compared to age-matched control group.
Table 8

Effect of pCMBS on the lens Ca\(^{2+}\) influx

Rat lenses were incubated in the medium used for the Ca\(^{2+}\) influx experiment with or without indicated concentrations of pCMBS. Incubation temperature was 21°C for 2 h. Lens calcium was determined by atomic absorption spectrophotometer. Mean values ± SE are reported.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ca(^{2+}) content (μmol/g dry wt)</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, None</td>
<td>4.37 ± 0.22</td>
<td>5</td>
</tr>
<tr>
<td>25 μM pCMBS</td>
<td>4.48 ± 0.60</td>
<td>4</td>
</tr>
<tr>
<td>50 μM pCMBS</td>
<td>5.89 ± 0.12*</td>
<td>4</td>
</tr>
<tr>
<td>75 μM pCMBS</td>
<td>6.66 ± 0.18*</td>
<td>4</td>
</tr>
</tbody>
</table>

* p < 0.01 compared to control.
**Table 9**

*Dithiothreitol effect on the Ca\(^{2+}\) influx in lenses from rats 48 h PI with selenite*

Lens Ca\(^{2+}\) influx experiments were determined as described in the section of methods. 2 mM DTT was added to the incubation medium. Lens calcium was determined by atomic absorption spectrophotometer. Mean values ± SE are reported.

<table>
<thead>
<tr>
<th>Group</th>
<th>Ca(^{2+}) content (μmol/g dry wt)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After incubation</td>
<td># of samples</td>
</tr>
<tr>
<td>Control</td>
<td>4.07 ± 0.22</td>
<td>9</td>
</tr>
<tr>
<td>Control DTT</td>
<td>4.24 ± 0.38</td>
<td>10</td>
</tr>
<tr>
<td>Selenite</td>
<td>7.03 ± 0.33*</td>
<td>9</td>
</tr>
<tr>
<td>Selenite DTT</td>
<td>5.65 ± 0.20**</td>
<td>10</td>
</tr>
</tbody>
</table>

* p<0.001 compared to control group.

** p<0.01 compared to selenite group.
calcium permeability could be partially reversed by the reducing agent, DTT.

2.2. Selenite effect on lens Ca\(^{2+}\)-ATPase activity - Lens homogenates were used to study the selenite effect on the Ca\(^{2+}\)-ATPase activity. About 50% inhibition of Ca\(^{2+}\)-ATPase activity from rats occurred in the lenses at 48 h after injection of selenite, and this inhibition was still apparent at 96 h after injection of selenite (Figure 11). A similar inhibition pattern was observed by using lens membrane vesicles as a source of Ca\(^{2+}\)-ATPase (Table 10). Lens membrane vesicles from the cortical region were also used for assay of Ca\(^{2+}\)-ATPase activity to provide information about localized inhibition of Ca\(^{2+}\)-ATPase from selenite-induced stress. At 48 h PI, there was less inhibition of Ca\(^{2+}\)-ATPase activity of the lens membrane vesicles as compared to the intact lenses (Figure 12). The lens Ca\(^{2+}\)-ATPase activity of the control group was 10.4 ± 1.5 Pi nmol/lens/h, the selenite group was 8.1 ± 1.3 Pi nmol/lens/h. Only about 22% inhibition of Ca\(^{2+}\)-ATPase activity occurred in the lens cortical region 48 h after administration of selenite (Table 11). These data indicate that there is a greater extent inhibition of Ca\(^{2+}\)-ATPase in the lens epithelial region. Due to the requirement for a large sample size (8 lens epithelia/sample), the direct quantification of Ca\(^{2+}\)-ATPase activity from the epithelial region was not carried out.

ATP-dependent Ca\(^{2+}\) uptake was studied by incubation of lens membrane vesicles in a solution containing 2 mM MgATP, 120 µM calcium and a trace
**Figure 11. Inhibition of lens Ca\(^{2+}\)-ATPase activity by selenite treatment.** Homogenates prepared from lenses of either age-matched control (■—■) or selenite-treated rats (▲—▲) at different times PI were used for the assay. Two lenses were pooled together as one samples. The values represent the means of 3 to 8 samples.
Table 10

Inhibition of lens membrane Ca\(^{2+}\)-ATPase by selenite treatment
- ATP hydrolysis and \(^{45}\)Ca\(^{2+}\) transport activity

Lens membrane vesicle preparations from control or selenite-treated rats were used in this study. Mean values ± SE are reported. Each sample was prepared from 4 - 6 lenses.

<table>
<thead>
<tr>
<th>Time, post-injection</th>
<th>Ca(^{2+}) nmol/lens/h</th>
<th>Pi nmol/lens/h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity</td>
<td># of samples</td>
</tr>
<tr>
<td>control</td>
<td>3.94 ± 0.29</td>
<td>13</td>
</tr>
<tr>
<td>24 h</td>
<td>1.95 ± 0.10*</td>
<td>4</td>
</tr>
<tr>
<td>48 h</td>
<td>1.01 ± 0.22*</td>
<td>5</td>
</tr>
<tr>
<td>72 h</td>
<td>1.19 ± 0.35*</td>
<td>4</td>
</tr>
<tr>
<td>96 h</td>
<td>0.81 ± 0.07*</td>
<td>5</td>
</tr>
</tbody>
</table>

* p<0.001 compared to control.
** P<0.05 compared to control.
Figure 12. Ca\(^{2+}\)-ATPase activity in lens homogenates and lens membrane preparations. Lens Ca\(^{2+}\)-ATPase was determined from whole lens homogenates, membrane preparations from whole lens as well as lens cortical region. Lenses from either age-matched control or selenite-treated rats after 48 h injection of selenite were used for the assay. 4 to 8 lenses pooled together as a sample, and at least 3 samples were used for each group.
Table 11

Ca^{2+}-ATPase activity in lens homogenates and lens membrane preparations

Lens Ca^{2+}-ATPase was determined from whole lens homogenates, membrane preparations from whole lens as well as lens cortical region. Lenses from either age-matched control or selenite-treated rats after 48 h injection of selenite were used for the assay. 4 to 8 lenses were combined as a sample.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>n</th>
<th>Selenite</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole lens homogenate</td>
<td>37.8 ± 3.6</td>
<td>4</td>
<td>19.2 ± 6.9</td>
<td>4</td>
</tr>
<tr>
<td>Whole lens membrane</td>
<td>18.4 ± 3.9</td>
<td>6</td>
<td>9.4 ± 2.4</td>
<td>3</td>
</tr>
<tr>
<td>Cortical membrane</td>
<td>10.37 ± 1.55</td>
<td>6</td>
<td>8.05 ± 1.32</td>
<td>6</td>
</tr>
</tbody>
</table>
amount of $^{45}\text{Ca}^{2+}$ at 37°C. ATP-dependent $\text{Ca}^{2+}$ uptake in lens membrane vesicles prepared from selenite-treated rats was significantly lower than that in the control membrane vesicles (Table 10). This inhibition of $\text{Ca}^{2+}$-uptake occurred as early as 24 h after injection of selenite, and still existed 96 h post-injection. However, in lens membrane vesicle preparations only 50% inhibition of $\text{Ca}^{2+}$-dependent ATP hydrolysis was observed at 48 h post-injection. It appears to be that the activity of $\text{Ca}^{2+}$ pump was more sensitive to selenite or selenite-induced stress than that of $\text{Ca}^{2+}$-dependent ATP hydrolysis.

In order to clarify whether or not the inhibition of lens $\text{Ca}^{2+}$-ATPase was due to the oxidation of -SH groups, the effect of DTT on $\text{Ca}^{2+}$-ATPase ATP hydrolysis and $\text{Ca}^{2+}$ transport activity in lens was examined. Lens membrane vesicles were preincubated with 5 mM DTT for 10-15 min at 37°C prior to the assay of $\text{Ca}^{2+}$-dependent ATP hydrolysis or ATP-dependent $\text{Ca}^{2+}$ uptake. DTT largely reversed the inhibition of $\text{Ca}^{2+}$-ATPase activity, but had no effect upon $^{45}\text{Ca}^{2+}$ transport (Figure 13). Therefore, it seems that oxidation of membrane -SH group by selenite treatment lead to inhibition of $\text{Ca}^{2+}$-dependent ATP hydrolysis activity.

2.3. Selenite effect on $\text{Na}^+$/Ca$^{2+}$ exchange in the rat lens - The effect of selenite on lens $\text{Na}^+$/Ca$^{2+}$ exchange activity was determined in lens membrane vesicles prepared from selenite-treated rats at different times following the injection of selenite. There was an approximately 40% decrease in $\text{Na}^+$/Ca$^{2+}$
Figure 13. DTT effect on Ca\(^{2+}\)-ATPase - ATP hydrolysis and \(^{45}\)Ca\(^{2+}\) pump activity of the rat lenses from selenite treatment. The membrane vesicle preparations were used for the assay. The membrane vesicle preparations were preincubated with 5 mM DTT for 10-15 min prior to Ca\(^{2+}\)-dependent ATP hydrolysis or ATP-dependent Ca\(^{2+}\) uptake assay. 6 lenses were pooled together as one sample, and 3-5 samples were used for the assay. ATP hydrolysis activity, Pi release (■——■); DTT pretreatment, Pi release (▲——▲); Ca\(^{2+}\) uptake (●——●); DTT pretreatment, Ca\(^{2+}\) uptake (□——□).
exchange activity in membrane vesicles prepared from lenses 48 h after injection of selenite (Figure 14 and Table 12). No obvious inhibition of Na\(^+\)/Ca\(^{2+}\) exchange activity was observed in the lenses taken at 24 h or 72 h after treatment of selenite (Table 12).

2.4. Selenite effect on Na\(^+\),K\(^+\)-ATPase activity - Lens Na\(^+\),K\(^+\)-ATPase activities were compared between age-matched control animals or selenite-injected animals (Table 13). Na\(^+\),K\(^+\)-ATPase activities were not significantly affected by selenite treatment.

3. Potential mechanism of selenite action

3.1. Lens epithelial membrane protein thiol blot - The possibility that oxidation of critical -SH groups occurs in lens membranes as a result of selenite treatment was determined using a thiol blot. Thiol blots were carried out as described in experimental procedures. Lens epithelial membrane proteins (15 \(\mu\)g) were isolated from rats 48 h after treatment with selenite, then separated by two dimensional gel electrophoresis. Afterwards, the gel was either transferred to a nitrocellulose membrane for thiol blot or stained with silver. There was no apparent change of lens membrane proteins 48 h after injection of selenite (Figure 15). The thiol blot showed no differences between control and selenite group (Figure 16).
Figure 14. Selenite effect on Na\(^+\)/Ca\(^{2+}\) exchange in rat lens membrane vesicles. Time-dependent \(^{45}\)Ca\(^{2+}\) uptake in the presence or absence of an outwardly oriented Na\(^+\) gradient at 25°C. Vesicles were prepared from at least 6 lenses from rats 48 h PI of selenite or age-matched control animals. The values represent means of 4 preparations. Control, (+) gradient (●—●); Se, (+) gradient (□—□); Control, (-) gradient (■—■); Se, (-) gradient (○—○).
Table 12

Relationship between Na⁺/Ca²⁺ exchange in lens membrane vesicles and time following selenite treatment

Rates were determined from the linear regression analysis of data from 0 to 60 sec Ca²⁺ uptake following transfer of vesicles to the buffer containing ⁴⁵Ca²⁺. Each sample was prepared from 6 lenses.

<table>
<thead>
<tr>
<th>Time after treatment</th>
<th>Number of samples</th>
<th>Rate of Na⁺/Ca²⁺ exchange (µmol Ca²⁺/min/mg proteins)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>24 h</td>
<td>3</td>
<td>1.2 ± 0.35</td>
</tr>
<tr>
<td>48 h</td>
<td>4</td>
<td>1.4 ± 0.14</td>
</tr>
<tr>
<td>72 h</td>
<td>3</td>
<td>1.0 ± 0.18</td>
</tr>
</tbody>
</table>

* Significantly less than age-matched control at p<0.05 level.
Table 13

$\text{Na}^+,\text{K}^+\text{-ATPase in rat lens}$

Lenses were removed from animals at different time intervals following the administration of selenite. Ouabain-sensitive ATP hydrolysis was quantified with lens homogenates. Two lenses were pooled together per sample. Values are means ± SE.

<table>
<thead>
<tr>
<th>Time, post-injection</th>
<th>Activity (πi nmol/mg protein/h)</th>
<th></th>
<th></th>
<th>Number of samples</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Number of samples</td>
<td>Selenite</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>147 ± 5.7</td>
<td>5</td>
<td>133 ± 6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>123 ± 20</td>
<td>7</td>
<td>125 ± 23</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>136 ± 4</td>
<td>4</td>
<td>120 ± 9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>96 h</td>
<td>132 ± 8.8</td>
<td>5</td>
<td>128 ± 13.8</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
Figure 15. Silver stained gel of lens epithelial cell membrane proteins separated by isoelectric focusing two dimensional electrophoresis. Gel A: Control. Gel B: selenite treated group, 48 h after injection of selenite.
Figure 16. Thiol blot on lens epithelial membrane proteins. Gel A. Control, Gel B. selenite-treated group, 48 h after injection of selenite. Regular lines on lower gel are artifacts of handling process.
3.2. Reactive species produced by GSH reaction with selenite

3.2.1. Reduction of NBT or cytochrome c by GSH and selenite - One-electron transfer reactions during selenite-dependent oxidation of GSH were studied using either NBT or cytochrome c reduction. The effect of SOD on the reduction of cytochrome c or NBT was used to detect the formation of superoxide ion. When both selenite and GSH were presented cytochrome c or NBT was rapidly reduced (Table 14, Table 15, Figure 17). The rates of both NBT and cytochrome c reduction were dependent upon selenite concentration (Figure 17). Under aerobic conditions a lag (75 seconds) occurred prior to the establishment of a linear rate of NBT reduction. SOD completely inhibited the NBT reduction. Further, the NBT reduction rate was doubled under anaerobic conditions and was not inhibited by SOD (Table 15). It appears that the intermediates formed in the selenite-dependent oxidation of GSH transferred electrons to NBT either directly or via superoxide radical. Catalase, only at very high concentration, inhibited the aerobic rate of NBT reduction induced by selenite. However, when both catalase and SOD were added to the reaction system containing GSH/selenite, no further inhibition of NBT reduction occurred as compared to SOD itself.

Due to the higher sensitivity of cytochrome c reduction compared to NBT reduction (Figure 17), cytochrome c reduction could be measured at much lower concentrations of selenite. The rate of cytochrome c reduction by GSH (1 mM) plus selenite (0.625 μM) was similar whether or not oxygen was
Table 14

GSH/selenite-dependent reduction of NBT

Reaction volume (2 ml) contained 100 μM NBT, 4 mM GSH, 15 μM selenite, and 50 mM potassium phosphate buffer with 10 mM EDTA at pH 7.4. SE from at least 3 determination was <10% of mean. Different amount of enzyme was added to this control reaction system.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Reduction Rate (ΔA560nm/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Control</td>
<td>0.323</td>
</tr>
<tr>
<td>Control, (-) Selenite</td>
<td>0</td>
</tr>
<tr>
<td>Control, (-) GSH</td>
<td>0</td>
</tr>
<tr>
<td>SOD (200 U)</td>
<td>ND</td>
</tr>
<tr>
<td>SOD (400 U)</td>
<td>0.398</td>
</tr>
<tr>
<td>SOD (800 U)</td>
<td>ND</td>
</tr>
<tr>
<td>Catalase (400 U)</td>
<td>0.430</td>
</tr>
<tr>
<td>Catalase (800 U)</td>
<td>ND</td>
</tr>
<tr>
<td>Catalase (400 U)/SOD (400 U)</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.
**Table 15**

*GSH/selenite dependent reduction of cytochrome c*

Reaction volume (2 ml) contained 4 mM GSH, 1 mM EDTA, 50 mM phosphate buffer (pH 7.4) with either 0.625 μM selenite and 10 μM cytochrome c, and indicated amount SOD was added to this control reaction system. Mean values ± SE are reported.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Reduction Rate (ΔA_{550 \text{nm/min}})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Control</td>
<td>0.057 ± 0.02</td>
</tr>
<tr>
<td>Control, (-) GSH</td>
<td>ND</td>
</tr>
<tr>
<td>Control, (-) Selenite</td>
<td>ND</td>
</tr>
<tr>
<td>SOD (400 U)</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.
Figure 17. Concentration dependence of cytochrome c and NBT reduction on selenite concentration. Reaction solutions contained 4 mM GSH and indicated concentration of selenite. Either 100 μM cytochrome c (■—■) or 100 μM NBT (●—●) was used for the assay at 25°C and pH 7.4. The linear rates were measured.
present. Further, SOD had no effect on the cytochrome c reduction (Table 16). These data indicate that cytochrome c reduction by GSH and selenite is not mediated by superoxide.

3.2.2. Effect of pH - In order to study the relevance of this free radical formation to the physiological pH, the effects of acidity on both NBT reduction and O₂ uptake was investigated. The rate of NBT reduction in the presence of selenite/GSH increased significantly between pH 7.0 and 8.0 (Figure 18A), but increased very slowly below pH 6.5. Under aerobic conditions, oxygen uptake was similarly affected by pH (Figure 18B).

3.2.3. Effect of free radical scavengers - Mannitol and Trolox C were used to determine whether or not free radicals were involved in NBT and cytochrome c reduction. Mannitol had no effect, but 10 mM Trolox C inhibited NBT reduction in both aerobic and anaerobic conditions (Figure 19). Due to the low solubility of FeCl₃ at higher pH, the FeCl₃ effect on NBT reduction was carried at pH 6.9. FeCl₃ (100-150 μM) inhibited the aerobic reduction of NBT in a concentration dependent fashion (Figure 20). These data indicated that NBT reduction by GSH and selenite is not mediated by hydroxyl radicals since mannitol is an effective scavenger of hydroxyl radical. The radical species generated by GSH and selenite can function as a reductant, and these radicals lead to reduction of Fe³⁺ to Fe²⁺.
Table 16

Effect of catalase and SOD on GSH/cysteine-dependent O₂ uptake

Reaction volumes (1.5 ml) contained either 1 mM GSH or 1 mM cysteine, 100 μM EDTA, 50 mM phosphate buffer (pH 7.4) and 20 μM selenite. Linear rates of O₂ uptake were recorded following the initial lag (2 min). The values represent means of at least three determinations. No O₂ uptake occurred when either thiol or selenite alone was present.

<table>
<thead>
<tr>
<th>Condition</th>
<th>O₂ uptake (nmol ml⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>GSH</td>
<td>7.65 ± 0.31</td>
</tr>
<tr>
<td>Cysteine</td>
<td>14.1 ± 0.77</td>
</tr>
</tbody>
</table>

ND, not determined.
Figure 18. Effect of pH on NBT reduction and $O_2$ uptake by GSH and selenite. NBT was reduced with 1 mM GSH, 15 $\mu$M selenite, 100 $\mu$M NBT in 50 mM phosphate buffer containing 100 $\mu$M EDTA (A). Oxygen uptake was quantified with 1 mM GSH and 32 $\mu$M selenite at 30°C (B). The results were expressed as nmoles $O_2$ consumed ml$^{-1}$min$^{-1}$. Rates of both NBT reduction and $O_2$ uptake were recorded after the initial lag period.
Figure 19. Factors affecting NBT reduction by GSH and selenite. Reaction solutions contained 4 mM GSH, 15 μM selenite and 100 μM NBT with indicated concentration of enzymes or free radical scavengers. The reaction buffer was 50 mM phosphate buffer (pH 7.4) with 1 mM EDTA. The values are reported as means of at least 3 determination.
Figure 20. Fe$^{3+}$ effect on NBT reduction by GSH and selenite. NBT (100 μM) was reduced with 1 mM GSH and 50 μM selenite, different concentration of Fe$^{3+}$ in 50 mM phosphate buffer (pH 6.9). The linear rate was recorded after initial lag.
3.2.4. Oxygen uptake by GSH and selenite - As observed for NBT reduction, there was an approximate 2 min initial lag period before linear, GSH/selenite-dependent uptake of \( \text{O}_2 \) was observed. Approximately 4 \( \mu \text{mol} \) of GSH were consumed for each \( \mu \text{mol} \) of \( \text{O}_2 \) consumed in the first 30 min of this reaction (Figure 21). In the absence of \( \text{O}_2 \), 25 nmol of selenite caused the consumption of 100 nmol GSH, compared to >400 nmol oxidized GSH in the presence of \( \text{O}_2 \).

Because some radicals can use \( \text{O}_2 \) as electron acceptor, the \( \text{O}_2 \) uptake by GSH reaction with selenite may be partially related to free radical generation. Therefore, different free radical scavengers were used to test their effect on \( \text{O}_2 \) uptake. Ascorbate, NBT, DMPO, and \( \text{FeCl}_3 \) inhibited GSH/selenite-dependent \( \text{O}_2 \) uptake (Figure 22). It appears that free radical species are partially responsible for the \( \text{O}_2 \) uptake by GSH and selenite. Catalase also inhibited the \( \text{O}_2 \) uptake by reaction of selenite with either GSH or cysteine. This indicates that \( \text{H}_2\text{O}_2 \) may be formed during the reaction of GSH or cysteine with selenite. The addition of either SOD or Trolox C caused an increase in \( \text{O}_2 \) uptake (Figure 22).

3.2.5. DNA damage induced by GSH and selenite - The potential reactivity of the reactive species generated by the reaction of GSH with selenite was determined using a DNA strand break assay. When plasmid DNA (pMAMneo-CAT) was exposed to 1 mM GSH and 25 \( \mu \text{M} \) selenite for 30 min, selenite-
Figure 21. Rate of selenite-dependent GSH oxidation and \( O_2 \) uptake. Reaction solution contained 1 mM GSH, 25 \( \mu \)M selenite in phosphate buffer at pH 7.4 with 100 \( \mu \)M EDTA. Reactions were carried out at 25°C. Oxygen uptake (△---△); aerobic GSH consumption (■—■); anaerobic GSH consumption (● --- ●). Means for at least 3 determination are reported. SE was <10% of mean.
Figure 22. Effect of electron acceptors on GSH/selenite-dependent O₂ uptake.
Reaction solution contained 1 mM GSH, 32 μM selenite and the indicated concentration of electron acceptor in phosphate buffer (pH 7.4) with 100 μM EDTA. The temperature for the O₂ uptake was 30°C.
dependent oxidation of GSH led to DNA damage as evidenced by the near-complete conversion of the supercoiled closed-circular plasmid DNA to a nicked form (Figure 23). The DNA damage effect was quantified as a decrease in the relative amount of supercoiled DNA. DNA damage was observed to be dependent on the concentration of both GSH and selenite (Figure 24). The addition of SOD failed to decrease the GSH/selenite-dependent DNA damage (Table 17), but additions of Trolox C, Fe$^{3+}$, or sodium azide significantly protected the DNA (Table 17). DNA damage also occurred under anaerobic conditions.

3.3. Deferoxamine (DF) effect on cataract formation induced by selenite

3.3.1. Lens Transparency - Treatment of rats with selenite alone was followed by formation of posterior subcapsular cataract (PSC) within 12-48 h and nuclear cataract (NC) within 72-96 h. At a level of 3.35 μmol DF/g body wt, there was 80% protection against the appearance of PSC after 48 h, and a 25% protection against NC after 96 h. About 20% had less severe nuclear cataract than that observed with selenite treatment alone (Figure 25), and the other 55% of the lenses had developed the typical nuclear opacity. At the age of 35-40 days (3 weeks post-injection), a cataract, involving both the nuclear and cortex, appeared in these "protected" lenses (clear lenses at 96 h PI). There was a 50% occurrence of exclusively cortical cataract in animals three weeks after treatment with DF alone. Slit lamp evaluation of eyes in rats, 48
Figure 23. Effect of GSH/Se on plasmid DNA (pMAMneo-CAT). DNA damage was determined by measuring the conversion of closed circular supercoiled DNA to an open circular form. The plasmid DNA concentration is 0.13 μg/10 μl. The reaction was carried out at room temperature. N = nicked, L = linear, S = supercoiled.

Lane 1: plasmid DNA in phosphate buffer (pH 7.4).
  CONTROL
Lane 2: plasmid DNA + 25 μM selenite.
  Se CONTROL
Lane 3: plasmid DNA + 1 mM GSH.
  GSH CONTROL
Lane 4: plasmid DNA + 1 mM GSH/25 μM selenite.
  COMPLETE
Lane 5: plasmid DNA + 1 mM GSH/25 μM selenite + 100 μM FeCl₃.
  Lane 6: plasmid DNA + 100 μM FeCl₃.
  Fe CONTROL
Figure 24. GSH or selenite concentration effect on the DNA damage induced by GSH/selenite. GSH/selenite-induced DNA damage was determined as described in Figure 24. (A) Variable GSH concentration in the presence of 25 µM selenite, and (B) variable selenite concentrations were used in the presence of 1 mM GSH.
Table 17

Effect of free radical scavengers on the GSH/selenite-mediated DNA damage

DNA damage was detected as a decrease in the relative amount of supercoiled DNA. Percents were detected from areas quantified by densitometric scans of a photograph. Values are the mean ± SE of 3-5 determinations. Percent DNA = (Area of supercoiled)/(Total area, nicked + supercoiled)

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>% Supercoiled DNA</th>
<th>% Protection*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS buffer</td>
<td>71.5 ± 5.5</td>
<td>-</td>
</tr>
<tr>
<td>GSH (1 mM)</td>
<td>73.3 ± 2.8</td>
<td>-</td>
</tr>
<tr>
<td>Selenite (25 µM)</td>
<td>72.3 ± 3.5</td>
<td>-</td>
</tr>
<tr>
<td>GSH/Se</td>
<td>23.2 ± 3.4</td>
<td>-</td>
</tr>
<tr>
<td>GSH/Se + SOD (100 U)</td>
<td>11.5 ± 6.0</td>
<td>0</td>
</tr>
<tr>
<td>GSH/Se + Trolox C (22 mM)</td>
<td>43.4 ± 0.7</td>
<td>41</td>
</tr>
<tr>
<td>GSH/Se + Mannitol (15 mM)</td>
<td>42.9 ± 0.9</td>
<td>40</td>
</tr>
<tr>
<td>GSH/Se + Fe³⁺ (100 µM)</td>
<td>50.0 ± 2.9</td>
<td>55</td>
</tr>
<tr>
<td>GSH/Se + Azide (10 mM)</td>
<td>54.5 ± 10</td>
<td>64</td>
</tr>
<tr>
<td>GSH/Se, Anaerobic</td>
<td>16.6 ± 6.0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Percent recovery was calculated using the relative % supercoiled DNA in control samples (72%) and fully damaged DNA (GSH/Se = 23%) in the calculation: % Protection = (% "protected DNA" -23%)/(% "control DNA" - 23%)
Figure 25. Effect of DF dose on the occurrence of cataract in selenite-treated rats. Rats were injected with 30 nmol selenite/g body wt along with the first of three injections of DF at the designated dose. PSC was examined 48 h after the treatment of selenite; NC was evaluated 96 h PI. At least 6 animals from two litters were compared with age-matched controls.
and 96 h post-injection was in accord with the result obtained by examination of extracted lenses.

3.3.2. Effect on lens glutathione - In agreement with early observations by Bunce and Hess (69), significant loss of total glutathione in lens occurred after injection of selenite. Concurrent treatment with DF did not affect total glutathione concentration up to 96 h after administration of selenite, but prevented the expected recovery of glutathione which occurs in lenses from selenite-treated rats (Figure 26). DF alone caused an initial 30% decrease in lens glutathione concentration, which returned to control values by 29 days after treatment.

3.3.3. Effect on lens ion homeostasis - In lenses from selenite-treated rats, total Ca$^{2+}$ concentration significantly increased 48-96 h PI. The concurrent administration of selenite and DF resulted in a significantly lower accumulation of Ca$^{2+}$ in these lenses 96 h post-injection when compared to selenite treatment alone. As a group, lenses from the animals treated simultaneously with selenite and DF tended to have more Ca$^{2+}$ than controls, but the difference was not statistically significant (Figure 27). However, this group consisted of three classes of lenses with following statistics (mean ± SE): clear lens 0.96 ± 0.01 μmol/g dry wt, partial protection 1.49 ± 0.2 μmol/g dry wt, and nuclear cataract 4.3 ± 0.6 μmol/g dry wt. As lenses in the selenite-
Figure 26. Effect of selenite and DF on glutathione content in rat lens. Data points represent the mean of at least three samples (1 lens per sample), from at least 2 different litters. Control (□——□); (+) selenite (■——■); (+) DF, (+) selenite (▲······▲); (+) DF (●——●). After treatment, (+) selenite, (+) DF, were different from controls 24 h after injection of selenite.
Figure 27. Effect of selenite and DF on lens calcium content. Data points represent the mean of at least three samples, 2 lenses/sample, each data point represents a different litter. Control (□——□); (+) selenite (■—■); (+) DF, (+) selenite (▲······▲); (+) DF (●—●). After 72 h only the calcium in lenses from (+) selenite-treated rats were significantly different from control. After 18 days only the calcium content in lenses from (+) selenite, (+) DF-treated animals was different from controls.
treated animals began to grow again, Ca$^{2+}$ concentration began to return to normal. In animals receiving both selenite and DF, Ca$^{2+}$ accumulation in the lens continued, so that the calcium content became 10-20 times higher than that of calcium concentrations in control lenses (Figure 27). The quantification of other ion concentration in the lens may provide the additional information about functional status of membranes, and also these ions may directly or indirectly affect the lens calcium homeostasis. The treatment of rats with both selenite and DF also resulted in a loss of Na$^{+}$ homeostasis (Figure 28) and a accompanying loss of K$^{+}$ homeostasis (Figure 29). Significant differences compared to controls were not observed in concentrations of either Na$^{+}$ or K$^{+}$ in lenses from animals treated with only selenite or DF (Figure 27-29). Also, there was a trend toward less lens K$^{+}$ with lens growth and development (Figure 29B).
**Figure 28. Effect of selenite and DF on lens sodium content.** Data points represent the mean of at least three samples, 2 lenses/sample; each value represents a different litter. Control (□ — □); (+) selenite (■ — ■); (+) DF, (+) selenite (▲ — ▲); (+) DF (● — ●). After 96 h, the sodium content only in the lenses from (+) DF, (+) selenite-treated rats (▲ — ▲) was significantly different from controls (□ — □). After 18 days this difference had increased to about 10 times the control level.
Figure 29. Effect of selenite and DF on lens potassium. Data points represent the mean of at least three samples, 2 lenses/sample; each data point represents a different litter. Control (□—□); (+) selenite (■---■); (+) DF, (+) selenite (▲···▲); (+) DF (●—●). Only after 18 days was the potassium content in lenses from (+) DF, (+) selenite-treated animals (▲···▲) significantly less than that of controls (□—□).
DISCUSSION

A single injection of selenite into 10- to 15-day old rats causes a 3- to 5-fold increase in lens calcium content (82). This increase in Ca\(^{2+}\) concentration occurs in the presence of normal concentrations of Na\(^{+}\) and K\(^{+}\) and before the formation of nuclear cataract (69). Recent studies indicate that the elevated lens calcium is a cause of nuclear cataract induced by selenite. The mechanism for selenite cataractogenesis involves Ca\(^{2+}\) activation of calpain II, with subsequent proteolysis, loss of soluble proteins and increase of lens insoluble proteins (64,71). Therefore, elucidation of the mechanism of selenite cataractogenesis requires study of the effect effect of this agent on calcium homeostasis.

Which components participate in lens calcium regulation?

As a first step, it is necessary to determine which processes are involved in lens calcium regulation. Like other tissues or cells, the regulation of lens calcium is achieved by restricted membrane permeability to calcium, and processes of active calcium extrusion. Calcium enters the lens principally through non-specific cation channels activated by membrane stretch (31). The existence in lens epithelial cells of Ca\(^{2+}\) channels similar to those found in excitable tissues such as nerve cells, has been suggested but not proven (42). Ca\(^{2+}\)-efflux mechanisms in the lens might depend on both Ca\(^{2+}\)-ATPase and Na\(^{+}\)/Ca\(^{2+}\) exchange. The occurrence of Na\(^{+}\)/Ca\(^{2+}\) exchange in the lens has
been a point of controversy. In our experiments we have observed both Na\textsuperscript{+} gradient-dependent Ca\textsuperscript{2+} uptake and release by lens membrane vesicles (Figure 5, Figure 6, Table 3, Table 4), substantiating the occurrence of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in rat lens. Data from experiments with intact lenses were consistent with observed responses using isolated lens membrane vesicles. The increase in lens Ca\textsuperscript{2+}, in the absence of a transmembrane Na\textsuperscript{+} gradient, implicated Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in regulating ion homeostasis in the lens (Table 5) since the driving force of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange is transmembrane Na\textsuperscript{+} gradient. The Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange system of other cells has been described as a low affinity, high capacity system, in contrast to the high affinity but low capacity of Ca\textsuperscript{2+}-ATPase (100). Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange may be of special importance at high Ca\textsuperscript{2+} concentration, since the contribution of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange to Ca\textsuperscript{2+} efflux became more apparent when lens Ca\textsuperscript{2+} concentration was increased (Table 6).

Typically choline or NMDG can be used as a cation substitute for Na\textsuperscript{+}. A different response in Ca\textsuperscript{2+} content occurred in lens when these cation substitutes were used (Table 5 and Table 6). The use of NMDG resulted in a more specific response in the intact lens. The use of choline, on the other hand, can cause a complex response in ion transport if cholinergic receptors exist (101). Also, choline stimulates Na\textsuperscript{+},K\textsuperscript{+}-ATPase when Na\textsuperscript{+} is suboptimal (9 mM Na\textsuperscript{+}) (102). Although the increased Ca\textsuperscript{2+} content in lens incubating in the absence of a Na\textsuperscript{+} gradient medium might result from non-specific effects on membrane permeability, no swelling occurred in these lenses. Further,
transparency of Ca\(^{2+}\)-loaded lenses was improved only in the presence of a Na\(^+\) gradient, and correlated with the Na\(^+\)-dependent return of Ca\(^{2+}\) to near normal concentration. Experiments on both lens membrane vesicles and whole lenses established that Na\(^+\)/Ca\(^{2+}\) exchange is an important factor in regulating lens Ca\(^{2+}\) homeostasis.

Lens membrane vesicles obtained from rat lenses are capable of ATP-dependent \(^{45}\)Ca\(^{2+}\) uptake (Figure 7). The spatial distribution of Ca\(^{2+}\)-ATPase activity within the rat lens was consistent with that found in rabbit, bovine and human lenses (44, 48). About the 30% of Ca\(^{2+}\)-ATPase activity in rat lenses was located in the epithelial region, and the remainder was found in the cortical region (Figure 9). About 40 nmol Pi/lens/h of Ca\(^{2+}\)-ATPase activity has been observed in this study; a similar value to that reported by Iwata of 47 nmol Pi/lens/h for 10-12 week-old rats (103). In summary, Ca\(^{2+}\) enters the lens through NSC or Ca\(^{2+}\) channel, and both Ca\(^{2+}\)-ATPase and Na\(^+\)/Ca\(^{2+}\) exchange are responsible for calcium extrusion from lens.

What is the effect of selenite treatment on each of the components responsible for lens calcium regulation?

**Lens Ca\(^{2+}\) permeability** - The accumulation of calcium in lenses from selenite-treated rats can result from either increased influx, decreased efflux or both. A principal route for Ca\(^{2+}\) entry into lens cells is through non-selective cation channels (NSC) (31). The results of Ca\(^{2+}\)-influx experiments suggested that
increased permeability to Ca$^{2+}$ is partially responsible for the accumulation of calcium in cataractous lenses induced by selenite (Figure 10 and Table 7). Further, this increased membrane permeability to calcium may result from the oxidation of membrane sulfhydryl groups, since DTT partially reversed the increase in Ca$^{2+}$-influx observed in lenses from selenite-treated rats (Table 9). Therefore, NSC may also be opened by sulfhydryl oxidation. The importance of membrane -SH groups in controlling lens membrane permeability was reported by Hightower (65). In agreement with Hightower, there was an increased Ca$^{2+}$-influx when lenses were treated with the sulfhydryl reagent $p$-CMBS (Table 8). In vitro studies with rabbit lenses suggested that there is an irreversible loss of lens membrane -SH groups after selenite treatment, leading to a subsequent increase in lens membrane permeability (80,81). Although there is a rapid decrease in lens GSH after administration of selenite, GSSG accumulation in the lens was not observed (69,73). Perhaps GSSG was transported out of the lens to the aqueous humor, where these molecules interact with lens membrane superficial -SH groups to form mixed disulfide bonds. Selenite may also directly or indirectly lead to oxidation of membrane protein -SH groups and subsequently cause the lens membranes to become leaky. Treatment with DTT significantly decreased Ca$^{2+}$-influx in the lenses from selenite-treated rats, but there was still remained about a 20% increase in Ca$^{2+}$-influx after DTT treatment (Table 9). Early studies showed that oxidized fatty acids can act as calcium ionophores (88). Since lipid
peroxidation occurs in rat lenses following administration of large doses of selenite (104), calcium may also enter lens through the pores formed by these lipid peroxidation products. It appears that the majority of increased Ca\(^{2+}\)-influx following selenite treatment is reversible and transient (Figure 10), and this response may be related to available repair mechanisms, or a rapid turnover rate for NSC proteins. The pores formed by lipid peroxidation products in the lens may partially account for the increased Ca\(^{2+}\)-influx observed following selenite treatment.

**Na\(^{+}\)/Ca\(^{2+}\) Exchange** - The rate of Na\(^+\) gradient-dependent Ca\(^{2+}\) uptake was decreased by 40% in lens membrane vesicles from rats 48 h after administration of selenite (Figure 14, table 9). The mechanism leading to this transitory decrease of Na\(^+\)/Ca\(^{2+}\) exchange activity is not clear, but may be related to the oxidative stress induced by selenite, with consequent membrane damage. An increase in lens lipid peroxidation product was observed after selenite treatment (104). It has been demonstrated that Na\(^+\)/Ca\(^{2+}\) exchange is sensitive to its membrane environment (105).

**ATPase** - Lens Ca\(^{2+}\)-ATPase activity was inhibited by selenite treatment (Figure 11 and Table 10); similar results were obtained with lens homogenates or membrane vesicle preparations. About 50% inhibition of total Ca\(^{2+}\)-ATPase activity occurred in the rat lenses 48 h after injection of selenite, although only 22% inhibition was found in the cortical region (Figure 12). These results suggested that there is a greater extent of inhibition of Ca\(^{2+}\)-ATPase activity
in the epithelial region. Furthermore, decreased ATP-dependent Ca\(^{2+}\) uptake was also observed in this study (Table 7). Since Ca\(^{2+}\)-ATPase is a sulphydryl enzyme (106), a reducing agent, DTT, was used to determine whether or not this inhibition of Ca\(^{2+}\)-ATPase was due to the oxidation of -SH groups. The hydrolytic function of Ca\(^{2+}\)-ATPase could be restored by DTT treatment, but not the Ca\(^{2+}\) pump activity (Figure 13).

Possible modifications may have occurred in lens Ca\(^{2+}\)-ATPase besides oxidation of -SH groups. It seems that oxidation of -SH groups led to inhibition of both Ca\(^{2+}\)-ATPase ATP hydrolysis ability as well as Ca\(^{2+}\) pump activity, and an other unknown modification inactivated Ca\(^{2+}\) pump activity exclusively. The latter modification was quite similar to the uncoupling modification of Na\(^{+},K^{+}\)-ATPase produced by hydrogen peroxide (55). The difference in the extent of Ca\(^{2+}\)-ATPase inhibition with regard to its ATP hydrolysis assay and \(^{45}\)Ca\(^{2+}\) uptake assay (Table 7), together with the failure of DTT to restore the Ca\(^{2+}\) transport activity (Figure 13), strongly suggested that an uncoupling modification of Ca\(^{2+}\)-ATPase occurs in the lenses from selenite-treated rats.

The observation that selenite inhibited Ca\(^{2+}\)-ATPase \textit{in vivo} differed from the lack of Ca\(^{2+}\) ATPase inhibition in rabbit lenses treated with selenite \textit{in vitro} as reported by Hightower and McCready (80, 81). Galvan and Louis observed that ATP-dependent Ca\(^{2+}\) uptake by calf lens membrane vesicles was inhibited 30\% by 1 mM selenite (47). The species and age difference and concentration of selenite used may account for these conflicts (80).
Oxidative inhibition of Ca\(^{2+}\)-ATPase has been demonstrated in rabbit lenses (67). This inhibition of Ca\(^{2+}\)-ATPase activity by hydrogen peroxide occurs at an oxidant concentration of 5 \(\times\) 10\(^{-6}\) M, an order of magnitude lower than that thought to be present in the aqueous humor of some cataract patients (107). Lipid peroxidation products can also lead to inhibition of Ca\(^{2+}\)-ATPase (108). An increase in malondialdehyde has been reported in selenite cataract (104). Selenite itself or selenite plus GSH, has been shown to be a potent inhibitor of Ca\(^{2+}\)-uptake of liver microsomes (109). Therefore, it seems that selenite metabolites, rather than selenite itself, could be responsible for the inhibition of lens Ca\(^{2+}\)-ATPase and its associated Ca\(^{2+}\)-pump function.

Since the driving force for Na\(^{+}\)/Ca\(^{2+}\) exchange is a transmembrane Na\(^{+}\) gradient, Na\(^{+}\),K\(^{+}\)-ATPase inhibition could indirectly diminish this driving force and lead to calcium accumulation in the lens. This study revealed that there was no obvious inhibition of lens Na\(^{+}\),K\(^{+}\)-ATPase activity from selenite-treated animals (Table 13). This is in conflict with the observation of Bergad and Rathbun that the activity of dog kidney Na\(^{+}\),K\(^{+}\)-ATPase was inhibited 43\% by \textit{in vitro} treatment with 1 mM sodium selenite (79). Hightower and McCready also suggested that the loss of ion homeostasis in rabbit lenses exposed \textit{in vitro} to 100 \(\mu\)M sodium selenite is due to the inhibitory effects on the Na\(^{+}\),K\(^{+}\)-ATPase, which induced a 30\% increase of lens Na\(^{+}\) prior to lens calcium Ca\(^{2+}\) accumulation (80, 81). The dose of selenite used in these two \textit{in vitro} experiments was much greater than that delivered to the lens in the \textit{in vivo}
model. Since no apparent progressive increase in sodium or water content occurred in lenses from selenite-treated rats (69,71), the inhibition of Na⁺,K⁺-ATPase was not a major factor in selenite-induced Ca²⁺ accumulation in vivo. The time sequence of selenite toxicity to the components responsible for lens calcium regulation can be summarized in Figure 30. It appears that more than one factor responsible for regulating calcium levels must be be affected in order for cause calcium to accumulate in the lense. These results also indicate that the lens has a tremendous compensatory ability for maintaining its calcium homeostasis.

*Potential mechanism of selenite action*

*Membrane critical -SH oxidation* - Selenite is capable of oxidizing sulfhydryl groups (72), and the selenite cataract is characterized by significant loss of lens glutathione. However, no overall accumulation of oxidized disulfide in proteins or mixed disulfide has been observed (69, 73). Therefore, it is possible that selenite initiates cataract formation by oxidizing a small class of critical sulfhydryl groups (71). Certainly, thiol groups can show different levels of reactivity according to their local environment within a protein. Oftentimes, thiol groups on the protein surface are the most reactive. The presence of a basic amino acid in close proximity to the -SH group which favors the dissociation of the proton, also increases its reactivity (92). It appears that some membrane proteins are more susceptible to thiol group oxidation than
Time, after injection of selenite treatment

Components affected by selenite treatment

24 h

50% inhibition of Ca\textsuperscript{2+} transport by Ca\textsuperscript{2+}-ATPase
100% increase in Ca\textsuperscript{2+} influx

48 h

75% inhibition of Ca\textsuperscript{2+} transport by Ca\textsuperscript{2+}-ATPase
50% inhibition of ATP hydrolysis by Ca\textsuperscript{2+}-ATPase
30% decrease of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange activity
67% increase in Ca\textsuperscript{2+} influx

72 h

75% inhibition of Ca\textsuperscript{2+} transport by Ca\textsuperscript{2+}-ATPase
50% inhibition of ATP hydrolysis by Ca\textsuperscript{2+}-ATPase
No increase in Ca\textsuperscript{2+} influx

96 h

Figure 30. Time sequence of selenite effect on components responsible for lens calcium regulation.
others. In this study the effect of reducing agent, DTT, on Ca\textsuperscript{2+}-ATPase (Figure 13) and Ca\textsuperscript{2+} influx (Table 9), supports this hypothesis. However, thiol blot experiments failed to detect the oxidation of -SH groups in lens epithelial membrane proteins (Figure 16). Perhaps these -SH oxidations really occur in a few critical groups, and can not be detected by the current approach. However, a recent study by Bjornstedt et al. showed that selenodiglutathione is a highly efficient oxidant (110). If the protein -SH group is modified to protein-S-Se-SG, it is likely that protein-S-Se-SG can react with other sulfhydryl reagents (see the following pathway). As a result, this kind of modification would still produce positive reaction in a thiol blot. If so, no obvious difference would have appeared between proteins from controls and selenite-treated rats.

Although silver stain is a very sensitive technique for protein detection, it seems that more spots or proteins appeared on thiol blot than silver stain (Figure 15, 16). Due to its amplification effect by enzyme catalyzed reaction in thiol blot, perhaps thiol blot is more sensitive in detecting proteins with free -SH group than silver stain.

![Chemical Reaction](image)

* MPB is -SH reagent used for thiol blot.
** P-SS-MPB is positive in thiol blot

*Formation of free radicals* - Lens NSC, Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange
were affected by selenite treatment, but how selenite affects these different components is still not clear. A recent study suggested that active oxygen can be generated by the reaction of selenite with reduced glutathione (74). The SOD inhibited-cytochrome c reduction or NBT reduction was used to detect the formation of $\text{O}_2^{*-}$ (94). NBT reduction by GSH and selenite was inhibited by SOD (Table 14), but not the reduction of cytochrome c (Table 15). NBT and cytochrome c reduction also occurred under anaerobic conditions (Table 14 and 15), while the free radical scavenger, Trolox C, inhibited NBT reduction under both aerobic and anaerobic conditions. It is likely that different intermediates, such as thyl or selenyl radicals, may be responsible for NBT or cytochrome c reduction. Our experiments showed that an increase in $\text{O}_2$ uptake accompanied GSH oxidation by selenite (Figure 21), and that addition of Trolox C, ascorbate, Fe$^{3+}$, DMPO, or 1.25 mM NBT inhibited $\text{O}_2$ uptake (Figure 22). The effect of free radical scavengers on $\text{O}_2$ uptake by GSH and selenite indicated that one or more free radical species of high reducting potential are responsible for $\text{O}_2^{*-}$ formation. Catalase also inhibited oxygen uptake by GSH and selenite (Table 16); therefore, the formation of $\text{H}_2\text{O}_2$ was suggested. The reactive free radical species can reduce Fe$^{3+}$ to Fe$^{2+}$ (Figure 20). Thus, Fe$^{2+}$ would be available to interact with $\text{H}_2\text{O}_2$ to form hydroxyl radicals. Using DNA strand breakage as an assay, the potential reactivity of these reactive species has been tested. The DNA damage induced by reaction of GSH with selenite was also oxygen-independent, and free radical scavengers such as Trolox C, mannitol and azide,
protected the DNA against damage, while SOD did not (Figure 23, Table 17). Since DNA damage was both selenite and GSH concentration-dependent (Figure 24), the reactive intermediates produced by the GSH reaction with selenite must have been responsible for the DNA strand nicks observed. Recent studies showed that lens DNA damage is an early phenomenon occurring during selenite cataract formation (75, 76), and that this DNA damage may be related to the free radical formation by the reaction of GSH with selenite. Although catalytic oxidation reaction of GSH by selenite has been known for forty years (72), the detailed mechanism of this reaction is still not quite clear. Recently, Seko et al. proposed the following pathway (Figure 31, top) to explain the formation of reactive oxygen species by GSH and selenite (74). Our studies revealed that other reactive species besides $O_2^{-}$ form during the reaction of GSH with selenite, and these reactive species include $H_2O_2$, and possibly thyl or selenyl radicals (Figure 31, bottom). The relationships between these reactive species is summarized in Figure 31. As reviewed by Wardman (111), glutathione has the capacity to form free radicals that may function either as strong oxidants (GS$^+$ or as strong reductants (GSSG$^{3-}$). The reactive species in the current study have the characteristic of a strong reductant, as do GSSG$^{3-}$, $SeO_3^{2-}$, and/or GSSe$^{3-}$. The following pathway has been proposed by incorporate these two radicals into a sequence for the initial formation GSSeSG (Figure 32). These reactive species are very toxic to biomolecules, and the selenite toxicity may be mediated by these reactive species. Further
Figure 31. Free radical formation during GSH oxidation by selenite. The pathway enclosed in solid rectangle was taken from Seko et al. (74).
2GSH $\rightarrow$ 2G$^-\ +\ 2H^+

GS$^-\ +\ SeO_3^{2-}\rightarrow\ GS^-\ +\ SeO_3^{3-}$

GS$^-\ +\ GS^-\rightarrow\ GSSG^-$

2H$^+\ +\ SeO_3^{3-}\rightarrow\ SeO_2^-\ +\ H_2O$

GSSG$^-\ +\ SeO_2^-\rightarrow\ SeO_2^{2-}\ +\ GSSG$

SeO_2^{2-}\ +\ 2H^+\rightarrow\ SeO\ +\ H_2O$

2GSH $+$ SeO $\rightarrow$ GSSeSG $+$ H$_2$O

Overall Reaction

$4GSH\ +\ 2H^+\ +\ SeO_3^{2-}\rightarrow\ GSSeSG\ +\ H_2O\ +\ GSSG$

Figure 32. Proposed pathway of the reaction of GSH with selenite leading to formation of GSSeSG.
electron spin resonance (ESR) work is needed in order to characterize these free radicals.

**DF effect on cataract formation induced by selenite** - As a consequence of selenite-catalyzed oxidation of GSH, O$_2^\cdot$ and H$_2$O$_2$ could be produced and would be available to interact with free iron to form cytotoxic hydroxyl radicals (Fenton reaction). If selenite toxicity was mediated by reactive oxygen species, then chelation of iron might reduce formation of hydroxyl radicals in the lens. Therefore, the effect of the iron chelator, deferoxamine (DF), on selenite-induced cataract was tested. DF protected 80% of rats against posterior-subcapsular cataract formation at 48 h post-injection and 25% against nuclear cataract at 96 h. However, these protective effects by DF were transient, and lenses from the rats treated with DF/Se still became opaque in both nuclear and cortical regions within 2-3 weeks post-injection. The protective effect of DF appeared to be related to the reactive species formed by the GSH reaction with selenite. Effectiveness of DF against the PSC suggested that production of oxidant species, with resulting localized osmotic swelling, may be an important factor during the earliest stages of selenite toxicity.

DF, however, did not prevent the usual selenite-induced decline in total GSH (Figure 26), and further, DF alone caused a 30% loss in lens GSH that persisted for 20 days. Under certain circumstances, DF may also act as a prooxidant, especially in the presence of an abundance of ascorbic acid (112). The current studies showed that a high dose of DF provoked cataract formation
over a longer time period. This cataractogenic effect of DF may be related to chelator-induced iron deficiency in the lens, or the toxicity of DF itself (113, 114). The simultaneous administration of DF and selenite diminished the amount of excess calcium accumulation at four days post-injection (Figure 27), suggesting that calcium perturbation in the selenite lens may be a result of the generation of cytotoxic free radicals. The antioxidants butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) have also been shown to protect against the nuclear cataract induced by selenite (85). Individual treatment with selenite or DF did not significantly affect \( \text{Na}^+ \) and \( \text{K}^+ \) content in the lens (Figure 28 and Figure 29). Simultaneous DF administration with selenite did not alter the lens ion concentration within 48 h, but at 96 h post-injection, imbalance of monovalent cations was apparent and became statistically significant after 2 weeks.

**Summary**

A proposed mechanism for selenite cataractogenesis is summarized in Figure 32. The toxicity of selenite to the lens may be mediated through its reaction with reduced glutathione, the oxidative consumption of GSH by selenite diminishes the ability of the lens to resist oxidative stress. Thus, by-products of the reaction of GSH with selenite such as \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \) or other unknown species, may lead to oxidative damage to lens proteins, lipids and DNA. As a consequence of oxidative stress, the function of lens, lens \( \text{Ca}^{2+} \)-
Figure 33. Possible mechanisms of selenite cataractogenesis. The components responsible for lens Ca\(^{2+}\) regulation that was affected by selenite were shown in the top dotted rectangle. The pathway at the bottom solid rectangle was taken from David et al. (64).
ATPase, non-selective cation channel (NSC) and/or Na\(^+\)/Ca\(^{2+}\) exchange may be impaired, leading to calcium accumulation in the lens. Calpain in the nuclear fibers is activated by the resulting accumulation of Ca\(^{2+}\), and soluble \(\beta\)-crystallin and other lens proteins are degraded. This process results in the formation of insoluble protein aggregates in the lens. These insoluble protein aggregates scatter light, the lenses would become opaque.
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Vita

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