Effect of Substituted Dibenzoxazepines on Levels of Reduced Glutathione and Potassium Ions in Lenses of Rabbits *In Vitro* and of Rats *In Vivo*

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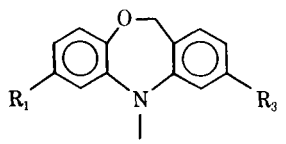
Abstract □ Levels of reduced glutathione and potassium ion were determined in rabbit lenses after incubation *in vitro* in the presence of various compounds. After rabbit lenses had been incubated *in vitro* with 4-[3-(7-chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepin-5-yl)propyl]-1-piperazineethanol hydrochloride (I), the levels of reduced glutathione and potassium ion were lower than after incubation with the corresponding 3-chloro analog (II). Compound I was also more potent than II in producing the hemolysis of rabbit erythrocytes. The loss of reduced glutathione and potassium ion from the lens, as mediated by I and 5-[(2-dimethylamino)ethyl]-5,11-dihydrodibenz[*b,e*][1,4]oxazepine maleate (IV), was correlated with the tendency of these compounds to bind to erythrocyte ghosts. Levels of reduced glutathione were also examined in the lenses of rats that had been given chronic doses in the diet of either of two substituted dibenzoxazepines (I or its trifluoromethyl analog, III). After being dosed with either compound, the lowest levels of reduced glutathione were found in the lenses of those rats that had developed morphological changes of the greatest severity. ^{14}C-Labeled compounds, both tricyclic and nontricyclic, were given orally to rabbits, and all of these compounds, whether or not they were cataractogenic, were found to be distributed in various portions of the eye. No chemical interaction was found between the substituted dibenzoxazepines and reduced glutathione when studies were conducted *in vitro*.

Keyphrases □ Dibenzoxazepines, substituted—effect on reduced glutathione and potassium-ion levels in rabbit and rat lenses, relationship to cataractogenicity □ Glutathione, reduced—possible interaction with substituted dibenzoxazepines, significance to cataractogenicity □ Cataractogenicity—effect of substituted dibenzoxazepines on levels of reduced glutathione and potassium ions in lenses of rabbits *in vitro* and of rats *in vivo* □ Potassium ions—effect of substituted dibenzoxazepines, rabbit and rat lenses

The isolated lens has been cultured successfully *in vitro* for considerable lengths of time (1). Conditions of culture employing rabbit lenses were described previously (2), and cataracts were produced by incubating rabbit lenses for 22 hr at 37° in a culture medium containing tyrosine and tyrosinase (2). The loss of reduced glutathione (GSH) from the lens has been found to occur during the formation of most types of cataracts, including those induced by drugs (1). 4-[3-(7-Chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepin-5-yl)propyl]-1-piperazineethanol hydrochloride (I), a substituted dibenzoxazepine, was reported to be cataractogenic in rats (3). In attempts to understand the mechanism of some dibenzoxazepine-induced cataracts, the loss of reduced glutathione and potassium ion from rabbit lenses was studied *in vitro*, as was the loss from the lenses of rats that had been fed chronic doses of some dibenzoxazepines. The results suggest that the measurement of reduced glutathione and potassium-ion concentrations in the lens may be useful in the preliminary evaluation of the potential cataractogenicity of substituted dibenzoxazepines (I–VII).



Compound	R_1	R_2	R_3
I	Cl	$-(CH_2)_3N\langle\rangle N(CH_2)_2OH$	H
II	H		Cl
III	CF_3		H
IV	H	$-(CH_2)_2N(CH_3)_2$	H
V	Cl	$-CONH_2$	H
VI	Cl	$-(CH_2)_2N(CH_3)_2$	H
VII	H		Cl

EXPERIMENTAL

Incubation of Rabbit Lenses *In Vitro* and Analysis of Rat Lenses—Female New Zealand albino rabbits (1.9–2.8 kg) were sacrificed by air embolism. Both eyes were excised, and the lens of each, with the capsule intact, was excised by a posterior approach. When possible, the two lenses from one rabbit were used in comparing the effects of two drugs or of one drug relative to an untreated control lens. The lenses were incubated individually in 10 ml of medium (2), which was oxygenated by bubbling a mixture of 5% CO_2 and 95% O_2 into the medium for 5 min after the drug had been added. The drugs were dissolved in saline at a concentration of 2×10^{-3} *M* and were added to the incubation medium to give, unless noted otherwise, a final concentration of 2×10^{-4} *M*. The medium contained: $MgSO_4$, 0.46 m*M*; Na_2HPO_4, 0.24 m*M*; KH_2PO_4, 0.25 m*M*; KCl, 3.33 m*M*; $NaHCO_3$, 23.1 m*M*; $KHCO_3$, 0.84 m*M*; glucose, 5.0 m*M*; NaCl, 129 m*M*; $CaCl_2$, 1.25 m*M*; and, for each 10 ml of medium, 1000 units of penicillin and 1 mg of streptomycin sulfate.

The incubation was carried out at 37° for 18 hr under an atmosphere of 5% CO_2 and 95% O_2. After incubation, the lenses were examined grossly for any damage to the epithelium or capsule. Intact lenses were then rinsed with distilled water (adhering fluid was removed by blotting with paper) and homogenized in 4.0 ml of water, using a Teflon–glass homogenizer. The protein of the lens homogenate (1.0 ml) was precipitated with 2 ml of 10% trichloroacetic acid. An aliquot of the supernate was used for the determination of reduced glutathione by the method of Ellman (4). The remaining supernate was used for the determination of the sodium-ion and potassium-ion contents with a flame photometer[1]. The data are expressed as percent loss of reduced glutathione relative to the control lenses. The relative sodium-ion and potassium-ion concentrations were calculated by normalizing the cation concentrations of the control lens to 1.0.

Sprague–Dawley rats[2] of both sexes were fed drug added to a diet of laboratory chow[3] from the time of weaning. The animals were sacrificed after they had developed cataracts of varying degrees of severity. Both lenses from one rat were analyzed in each single determination of reduced glutathione; the samples were

[1] Model 143, Instrumentation Laboratory, Inc.
[2] Charles River CD.
[3] Rockland rat chow.

Table I—Levels of Reduced Glutathione in Rabbit Lenses as a Function of Age after Treatment with I *In Vitro*

Body Weight, kg	Reduced Glutathione Content, μg/Lens ± *SE*: Incubated without Drug	Incubated with I[a]	Decrease from Control, %
1.9	972 (1)[b]	538 ± 22 (3)[b]	44.6
2.0	886 ± 52 (3)	422 ± 52 (4)	52.4
2.1	865 ± 26 (3)	402 ± 37 (2)	53.5
2.2	995 ± 67 (7)	498 ± 28 (3)	49.9
2.3	973 ± 7 (2)	787 ± 29 (3)	19.1
2.5	1141 ± 38 (3)	848 ± 34 (2)	25.7
2.6	1245 ± 39 (2)	751 ± 5 (2)	39.7
2.8	1340 (1)	944 (1)	29.6

[a] Final concentration of 2×10^{-4} *M*. [b] The number of animals is indicated in parentheses.

processed in the same manner as were rabbit lenses that had been incubated *in vitro*.

Purity and Specific Activity of ^{14}C-Labeled Compounds—Each of two compounds, administered to rabbits to study their distribution in the eye, was labeled with ^{14}C in some portion of the side chain. The chemical names, radiochemical purities, and specific activities, respectively, were: Compound I, 4-[3-(7-chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepin-5-yl)propyl]-1-piperazine-^{14}C-ethanol hydrochloride, 96%, 21.6 μCi/mg; and Compound IV, 5-[(2-dimethylamino)ethyl-1-2-^{14}C$_2$]-5,11-dihydrodibenz-[*b,e*][1,4]oxazepine maleate, 99%, 1.77 μCi/mg.

Distribution of Drugs in Rabbit Eye—Female New Zealand albino rabbits (2.5–2.6 kg) were given by gavage a 10-mg/kg dose of either I-^{14}C or IV-^{14}C. Blood was removed from an ear vein, and the radioactivity was determined by digesting 0.2 ml of sample in 0.5 ml of 1 *N* NaOH at 80° overnight. The sample was then bleached with 30% H_2O_2, neutralized with 0.2 ml of 2-ethylhexanoic acid, and added to 15 ml of scintillator (5).

The eye was excised at the time of sacrifice, and aqueous humor was aspirated from it with a syringe fitted with a 25-gauge needle. The entire lens, a portion of the cornea or of the combined retina, choroid, and sclera, and some of the aqueous or vitreous humor were analyzed by solubilizing each sample in sufficient solubilizer[4] and counting it in 15 ml of toluene scintillator; the latter preparation contained 5 g 2,5-diphenyloxazole and 0.3 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene/liter toluene.

Interaction *In Vitro* between Drugs and Reduced Glutathione—All drugs were added to phosphate-buffered saline, pH 7.4, at a concentration of 3×10^{-3} *M*. The incubation of compounds with reduced glutathione was carried out for 80–90 min at 3–6° to reduce the autoxidation of reduced glutathione. *N*-Ethylmaleimide and *p*-chloromercuriphenylsulfonic acid were chosen as reference compounds, since both react directly with —SH groups. At the end of the incubation, the reduced glutathione content of each sample was determined. The results obtained with samples containing drug were compared with those of a control sample that contained reduced glutathione but no drug.

Assay for Hemolysis—Fresh heparinized erythrocytes were obtained from female New Zealand albino rabbits (1.5–2.0 kg). The packed erythrocytes were washed three times with an equal volume of saline and were finally resuspended in an equal volume of saline. The incubation mixture contained 1.0 ml of a 50% suspension of erythrocytes and drug dissolved in 9 ml of saline buffered with 0.15 *M* phosphate at pH 7.4. All samples were incubated at 37° for 2 hr with intermittent shaking. At the end of the incubation, the erythrocytes were sedimented and the supernate was assayed spectrophotometrically for the amount of hemoglobin released (6). The results were expressed as percent hemolysis, using as 100% the absorbance obtained after having added erythrocytes to water at a final concentration of 5%.

Binding to Erythrocyte Ghosts—Rabbit erythrocyte ghosts were prepared by the method of Dodge *et al.* (7). Binding to erythrocyte ghosts was carried out by following spectrophotometrically the disappearance of drug from an incubation medium consisting of a 10% suspension of erythrocyte ghosts in 9.0 ml of 0.033 *M* sodium phosphate buffer, pH 6.0, and 1.0 ml of an aqueous solution of drug. Incubation was carried out, with shaking, at 25° for 30 min. After incubation, the erythrocyte ghosts were sedimented at 18,000×*g* for 30 min and the supernate was assayed spectrophotometrically at the wavelength of maximal absorption for each compound, *i.e.*, 212 nm for IV and 223 nm for I; the amount of each drug bound was expressed as nanomoles bound per milligram dry weight of ghost preparation.

[4] NCS Amersham/Searle.

Table II—Effect of Substituted Dibenzoxazepines on Loss of Reduced Glutathione from Rabbit Lenses *In Vitro*

Compound	Mean Reduced Glutathione Level, μg/Lens ± *SE*	Decrease of Reduced Glutathione[a], %
I	400 ± 43	58 ($p < 0.001$)[b]
IV	568 ± 47	41 ($p < 0.001$)
V	891 ± 68	7 (N.S.)[c]
Control (no drug)	963 ± 48	(0)

[a] *N* is equal to 5 for each compound. The rabbits weighed 1.9–2.2 kg. [b] Concentrations of reduced glutathione in the lenses after treatment with I were significantly lower ($p < 0.05$) than after treatment with IV. [c] N.S. = not significant.

RESULTS

The levels of reduced glutathione in rabbit lenses that had been incubated *in vitro* were found to be dependent on age (Table I). The lenses of female rabbits that weighed 1.9–2.2 kg (about 8 weeks old) contained lower levels of reduced glutathione than did those of female rabbits that weighed 2.5–2.8 kg (about 10 weeks old). In addition, the loss of reduced glutathione found after incubation *in vitro* with I was considerably greater in the lenses of younger rabbits than in those of older rabbits (Table I); the percent decrease from the control value changed abruptly in female rabbits that had attained the weight of 2.3 kg (about 9 weeks old).

Several dibenzoxazepines (I, IV, and V) were examined for their ability to affect the levels of reduced glutathione in rabbit lenses incubated *in vitro* (Table II). Levels of reduced glutathione in lenses that had been incubated with I or IV were significantly lower than those of the control lenses incubated without any drug. However, the loss of reduced glutathione in lenses incubated with I was greater than those incubated with IV. Incubation of lenses with V did not result in a loss of reduced glutathione.

To determine whether membranes of the lens had been affected by incubation with the dibenzoxazepines, the loss of potassium ion was determined in rabbit lenses incubated with the same compounds that had been employed in the experiment of Table II. The results (Table III) indicate that I, the compound that had produced the greater loss of reduced glutathione, also produced the greater loss of potassium ion. Levels of sodium ion were simultaneously determined in the same samples. The corresponding data for sodium ion (not shown) demonstrated a consistent elevation of sodium ion inside the lens in opposition to the outflow of potassium ion.

The binding of I and IV to rabbit erythrocyte ghosts, a well-characterized biological membrane, was also studied (Table IV). The results show that I, which produced a greater loss of reduced glutathione from rabbit lenses *in vitro* than did IV, was also bound more strongly to erythrocyte ghosts than was IV.

Two halogenated dibenzoxazepines were chosen for a more detailed study of the effect of tricyclic agents on the loss of reduced

Table III—Relative Concentrations of Potassium Ion in Rabbit Lenses after Incubation with Substituted Dibenzoxazepines *In Vitro*

Compound[a]	Experiment 1	Experiment 2	Mean
I	0.54	0.46	0.50
IV	0.73	0.52	0.63
V	0.94	0.89	0.92
Control (no drug)	(1.0)	(1.0)	(1.0)

[a] Drugs were present at 2×10^{-4} *M*. The rabbits weighed 2.2–2.8 kg.

Table IV—Binding of I and IV to Rabbit Erythrocyte Ghosts

Compound	Experiment	Nanomoles Bound per Milligram Dry Weight at 1.5×10^{-4} M	3.0×10^{-4} M	6.0×10^{-4} M
I	1	81.7	177	281
	2	81.7	190	327
IV	1	13.1	45.8	101
	2	0.0	35.9	88.2

glutathione and potassium ion from rabbit lenses *in vitro*. The two compounds, I and II, differ from each other in that the former contains a chloro substituent in the 7-position whereas the latter has it in the 3-position. Rabbit lenses were incubated *in vitro* with each compound at several concentrations (Fig. 1). The results show that both I and II stimulated the loss of reduced glutathione and potassium ion from the lenses; however, for both parameters, I, the 7-chloro-substituted compound, produced the greater effect. It is noteworthy that: *(a)* the curves for both reduced glutathione and potassium ion are sigmoidal; *(b)* the percent decrease of reduced glutathione *and* potassium ion is greater, at a given concentration, for I than it is for II; and *(c)* the loss of both reduced glutathione and potassium ion occurred initially in the presence of approximately the same concentrations of the two tricyclic compounds tested, I or II.

As an indication of the surface-active properties of some substituted dibenzoxazepines, the test compounds were incubated with rabbit erythrocytes (Fig. 2). Hemolytic activity was particularly notable for the piperazine-containing compounds, I and II, with the former being more potent than the latter. The dose-response curve for I is clearly sigmoidal. Studies with 7-chloro-5-[2-(dimethylamino)ethyl]-5,11-dihydrodibenz[*b,e*][1,4]oxazepine hydrochloride (VI) and the corresponding 3-chloro analog (VII) indicate that the 7-chloro-substituted compound was more hemolytic than the 3-chloro-substituted compound. However, here the difference was not as great as that observed in studies with the piperazine-containing compounds, because VI and VII are much less hemolytic than I and II.

The loss of reduced glutathione from rabbit lenses incubated *in vitro* with I may be correlated with a similar effect on the lenses of rats fed chronic doses of I or its trifluoromethyl analog (III) (Table V). In general, the amount of reduced glutathione lost from the lenses of these rats was proportional to the extent of the morphological change toward opacity.

Compound I is cataractogenic in rats (3) whereas IV is not[5]. To determine whether a compound that did not induce the formation of cataracts had, nevertheless, been distributed to various parts of the eye, the radioactive dibenzoxazepines (I and IV)

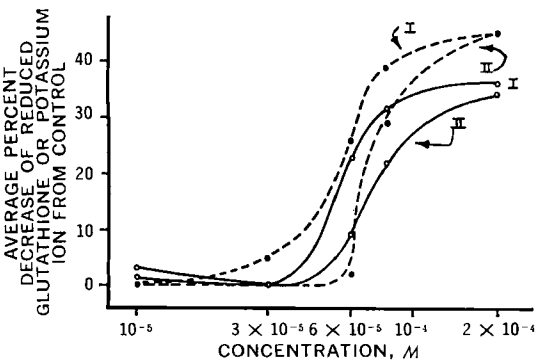


Figure 1—*Levels of reduced glutathione* (—) *and potassium ion* (- - -) *in rabbit lenses that had been incubated* in vitro *with various concentrations of a substituted dibenzoxazepine (I or II). Each point represents the average of values obtained from the individual incubation of three lenses.*

[5] Unpublished data, Toxicology Department, Squibb Institute.

Table V—Effects of I and III on Loss of Reduced Glutathione from Rat Lenses

Rat Number	Reduced Glutathione, μg/Lens	Decrease of Reduced Glutathione, %	Morphological Change
		I[a]	
10 Control rats (no drug)	79.0 ± 3.9	(0)	—
2	78.7	0	Faint anterior cortical striations
132	53.4	32	
54	39.9	50	Anterior and posterior cortical striations and/or pinpoint cataract
57	59.9	24	
93	54.4	31	
48	70.4	11	
83	37.1	53	$^1/_2$–$^3/_4$ opacity
120	15.6	80	
129	0.0	100	Entire lens opaque
146	20.0	75	
41	8.0	89	
		III[a]	
Five control rats (no drug)	68.6 ± 8.7	(0)	—
362	61.8	10	None
374	53.2	22	
358R[b]	28.9	58	$^1/_2$–$^3/_4$ opacity
348	20.9	70	Entire lens opaque

[a] Rats received 65 mg/kg/day in the diet. [b] Animal did not receive the drug for 18 weeks before it was sacrificed and its lenses were analyzed.

(Table VI) were administered to rabbits by gavage. Each compound appeared to be well absorbed, as shown by the levels of radioactivity in the blood. Furthermore, each compound and/or its metabolites were present in all portions of the eye. Similar results (not shown) were obtained in studies with two other nontricyclic compounds, 2-ethoxy-*N*-[2-(methylphenethylamino)-ethyl]-2,2-diphenylacetamide hydrochloride and 5-(2-dimethylaminoethyl)-1,3-dihydro-2-phenyl-1,5-benzothiazepin-4(5*H*)-one; like IV, these latter compounds do not produce cataracts in rats[5]. Thus, it appears that the distribution *per se* of I or its metabolites in various portions of the eye is not the only factor responsible for producing a cataract.

One way in which reduced glutathione could have been lost from the lens might be by means of the sodium-ion- and potassium-ion-dependent adenosine triphosphatase cation pump. It is known that gradients of sodium and potassium ions are maintained between the lens and its surroundings by the sodium-potassium-ion-dependent adenosine triphosphatase (8). Moreover,

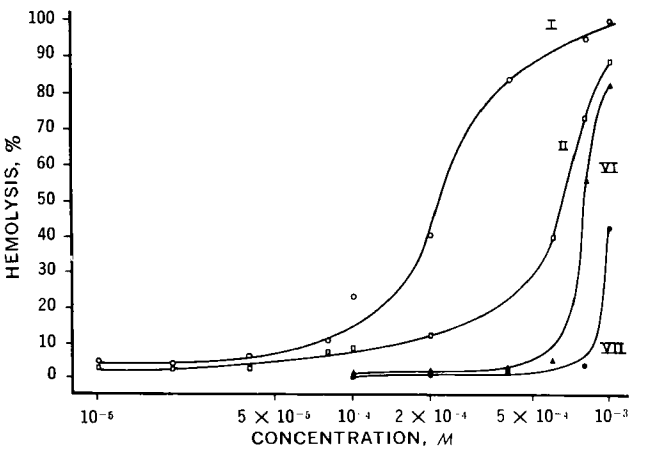


Figure 2—*Hemolytic activity of 7-chloro- and 3-chloro-substituted dibenzoxazepines as a function of their concentrations.*

Table VI—Concentrations of I and IV, Their Metabolites, or Both in Blood of Rabbits and Their Distribution in Various Parts of the Eye[a]

Concentration in Blood, hr	Compound, μg Equivalents/ml I	IV
1st	1.64	2.18
2nd	2.12	8.73
4th	2.24	1.57
Concentration in Tissue	**Compound, μg Equivalents/g I**	**IV**
Vitreous humor	0.124	0.101
Aqueous humor	0.056	0.164
Lens	0.079	0.089
Cornea	0.241	0.450
Combined retina, choroid, and sclera	0.739	0.853

[a] Each female rabbit (2.5–2.6 kg) received 10 mg/kg of drug in aqueous solution by gavage. The rabbits were sacrificed 4 hr after dosing.

rabbit lenses cultured *in vitro* are able to transport potassium ion (9). The present studies *in vitro* with rabbit lenses showed that ouabain, a specific inhibitor of the cation pump (10), did not produce any loss of reduced glutathione whereas it did produce an outflow of potassium ion (data not shown). Other studies have shown that reduced glutathione is lost from the lens by a prior conversion to oxidized glutathione, which is transported unidirectionally to the outside of the lens[6]. Thus, on this basis it appears that the cation pump is not responsible for producing the loss of reduced glutathione from the lens.

Another way in which reduced glutathione could have been lost from rabbit lenses *in vitro* might be by a direct chemical interaction between reduced glutathione and the substituted dibenzoxazepines. Accordingly, I, IV, or V was mixed with reduced glutathione in the molar proportions of 1:1 or 2:1. None of the incubations containing either a dibenzoxazepine and reduced glutathione or reduced glutathione alone showed the disappearance of any reduced glutathione. By contrast, in control experiments, none of the sulfhydryl-reactive material was recovered after reduced glutathione was incubated with *N*-ethylmaleimide or *p*-chloromercuribenzenesulfonic acid.

DISCUSSION

A number of investigations have shown that alterations in morphology or permeability of the lens epithelium can affect reduced glutathione or cation levels in the lens. In X-ray-induced cataracts, a progressive loss of reduced glutathione has been observed (11). X-irradiation is also known to produce morphological lesions of the lens epithelium (12) and an increase in permeability to cations in rabbit lenses either *in vitro* or *in vivo* (13). Kinsey and Reddy (14) reported that the lens epithelium actively transports potassium ion into the lens. More recently, Reddy *et al.* (15) demonstrated that the epithelium of cultured rabbit lenses, but apparently not the capsule, serves as the barrier to the efflux of reduced glutathione from the lens. In addition, a process for the degradation and synthesis of reduced glutathione in the lens was also indicated.

Studies with rabbit erythrocytes have shown that some dibenzoxazepines have surface-active properties. A similar phenomenon appears to be occurring in the rabbit lens *in vitro* since *(a) both* reduced glutathione and potassium ion are lost from the lens, and *(b)* those dibenzoxazepines that produce the greatest loss of reduced glutathione also produce the greatest loss of potassium ion. The sigmoidal dose-response curve for the loss of reduced glutathione and potassium ion from rabbit lenses and for the hemolysis of rat erythrocytes *in vitro* indicates that a threshold concentration of a compound is necessary before an effect on the lens epithelium or erythrocyte membrane becomes manifest. The failure to find any evidence for a chemical interaction of reduced glutathione and the substituted dibenzoxazepines and the apparent noninvolvement of the sodium–potassium-ion-dependent adenosine triphosphatase in maintaining endogenous levels of reduced glutathione are consistent with the suggested role of the lens epithelium. Thus, it is concluded that the surface-active properties of some substituted dibenzoxazepines are probably responsible for the loss of reduced glutathione and potassium ion from rabbit lenses *in vitro* and of reduced glutathione from rat lenses *in vivo*.

In view of Harding's (16) observation on the variability of endogenous levels of lens reduced glutathione in humans, rats, rabbits, and cattle as a function of age, the observation that rabbit lenses differ in their response to I as a function of age does not seem so surprising. Since these studies employed only the lenses of female rabbits, it is not known whether the lenses of male rabbits respond in a similar manner.

The change in the reduced glutathione levels of rat lenses produced by substituted dibenzoxazepines *in vivo* apparently does not precede a morphological change. Nevertheless, the test described, employing rabbit lenses *in vitro*, may be useful in screening compounds for their potential cataractogenicity. An additional parameter such as the water content of the lens, which was not measured in these studies, also could be included as a part of the assessment. The fact that a compound produces a loss of reduced glutathione and potassium ion from rabbit lenses *in vitro* does not imply that it must give rise to cataracts *in vivo*. However, a positive result in a test *in vitro* can alert the veterinarian and clinician to the need for routine examination of the eye for lenticular changes.

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