Chapter 2

Materials and Methods

Experimental Animal

Both sexes of Wistar albino rats were used in the study. Healthy male and female Wistar rats were obtained from Veterinary College, Mannuthy, Trichur, and reared in our animal house. The experimental and age-related control rat pups with their mothers were fed on standard rat feed (Hindustan Lever Ltd., Calcutta) and water ad libitum.

Cataract-Inducing Agent

Sodium selenite, 99% pure (Na$_2$SeO$_3$) A. R. (kemphasol), M. W. 172.95, was used as the cataractogenic agent throughout the investigation. Cataract was induced by subcutaneous injections of Na$_2$SeO$_3$ of various standardised dosages in the scuff of Wistar rat pups of 8 to 14th day post partum. The multiple low dosage groups and single high dosage groups were considered as the chronic and acute dosage groups respectively, during the present investigation.

Part I

Cataractogenic Potency of Sodium Selenite

The cataractogenic potency of selenite was studied in Wistar rat pups, of various age groups, and with varied doses of sodium selenite.
Age-Dependent Effects

The age groups used in this study varied from eight to fourteen days post partum. In each group, six to ten healthy rat pups (~20 g. wt.) were used. On the respective days post partum, the animals in each group were administered with a standardised dose of either multiple low dosage, or single or multiple high dosage sodium selenite subcutaneously. The low dosages were 4.8 µg., 9.6 µg., 14.4 µg., 19.2 µg., 24 µg. and 28.8 µg. per rat pups. The single and multiple high dosages were 32 µg., 40 µg., 48 µg., 56 µg., 64 µg., 72 µg., 80 µg., 88 µg. and 96 µg. per rat pups respectively. In the case of multiple doses, the administration continued on consecutive days after the first injection. On the 16th day post partum, or if the final dosage was given after the 16th day, the day after the final dosage, rat pups eyes were dilated with one per cent atropfine, and were examined ophthalmoscopically, to observe the cataractogenic frequency. In all the cases, distilled water (vehicle) treated rat pups of the respective age, served as controls. The frequency of cataractogenesis, mortality rate and body weight loss were tabulated.

Dosage-Dependent Effect

The dosage effect of multiple low dosage, was studied in healthy rat pups (~20 g. wt.), starting from day eight after birth. In the case of single or multiple high dosage, the administration started from day 10 post partum subcutaneously. The number of animals in each group ranged from six to ten.

The low dosages were 4.8 µg., 9.6 µg., 14.4 µg., 19.2 µg., 24 µg. and 28.8 µg. per rat pups respectively. The single and multiple high doses
included 32 µg., 40 µg., 48 µg., 56 µg., 64 µg., 72 µg., 80 µg., 88 µg. and 96 µg. per rat pups respectively. The multiple doses were given on consecutive days after the first dose. The number of doses for multiple low dosage group ranged from two to ten, starting from day eight post partum and for multiple high dosage group, the number of doses ranged from one to three, starting from day 10 post partum. The frequency of cataractogenesis, mortality rate and body weight loss were tabulated. In all the cases, distilled water (vehicle)-treated rat pups of the same age served as controls.

**Anti-Cataractogenic Potency of Vitamin-E**

Anti-cataractogenic effect of Vitamin-E was studied with a single optimised dose of selenite (32 µg./~25 g. rat pup) subcutaneously, with varied doses of Vitamin-E (α-tocopherol acetate-Merck, water soluble preparation) such as 1.25, 2.50 and 3.75 mg./rat pup subcutaneously. A 32 µg. selenite dosage was administered on 10th or 12th day aged rat pups subcutaneously, ten minutes later, a vitamin-E dosage was administered subcutaneously at another site. The administration of vitamin-E continued on consecutive days depending upon the dosage, with a maximum of five doses. The frequency of cataractogenesis and mortality rate were tabulated. In all the cases, non-vitamin-E injected, selenite injected rat pups of the same age served as controls.
Part II

Histomorphological Studies

Histomorphological studies were conducted on selenite cataract produced by an optimised dosage of 40 µg./rat pup subcutaneously on various stages after selenite administration. This includes one, two, three, four and five days after selenite treatment and the different stages after cataract development, that is, nuclear, mature and hyper mature stages. In all the cases vehicle treated rat pups of the same age served as controls. Vitamin-E + selenite-administered non-cataractous and cataractous lenses were also studied. In this case, selenite treated cataractous lenses of the same age served as controls.

Cataract Induction

Cataract was induced in Wistar rat pups by injecting a single dose of sodium selenite 40 µg./20 g. rat pups subcutaneously, on the 10th day post partum. In the case of vitamin-E+selenite treated lenses, 2.50 mg. dosage of vitamin-E, five doses were administered subcutaneously, starting on the 12th day after birth, along with a 32 µg./25 g. rat pup dosage as described earlier and the study was conducted after the final dose of vitamin-E.

The cataractous animals were sacrificed to study the morphological changes at various stages after selenite administration, and on 16th day post partum for selenite +vitamin-E administrated cases. All morphological studies were done in comparison with their age-related controls, using light microscopy and scanning electron microscopy.
Light Microscopy

The experimental animals and their controls were decapitated, and the complete eye lenses were dissected out by the posterior approach.

Materials needed

1) Carnoys fixative
   - Ethyl alcohol - 60 ml.
   - Chloroform (Merck) - 30 ml.
   - Glacial acetic acid (Merck) - 10 ml.
   Mixed well to get a 100 ml. fixative and stored in an air tight bottle.

2) Graded alcohol series - 70%, 80%, 90% and 100%

3) Clearing agent - Cedar wood oil (Nice)

4) Xylene (Qualigens)

5) Paraffin wax (58-60°C) (Merck)

6) Ehrlichs haematoxylin: Haematoxylin (Merck) 8 g. was dissolved in 240 ml. of absolute alcohol. In another beaker 24 g. potassium alum (aluminium potassium sulphate–BDH) was dissolved in 120 ml. distilled water by heating and 120 ml. glycerol (Merck) was added, cooled, and the haematoxylin solution was added in small quantities while shaking it well between each addition. Finally, 12 ml. of glacial acetic acid was added, and shaken well. It was ripened in bright sunlight for six to eight weeks before use.

7) Eosin (Glaxo): 1 g. eosin was dissolved in 100 ml. of distilled water and two to three small crystals of thymol (Merck) were added, and stored.

8) Acid-alcohol: 1% HCl (Merck) in 70% alcohol.
Procedure

The enucleated eye lenses were immediately fixed in Carnoy’s fixative at room temperature. One hour after fixation, the lenses were cut into equal halves, anterio-posteriorly or meridionally and kept in the fixative overnight.

Fixed lenses were transferred to 70% ethyl alcohol. Dehydration was carried out in ascending grades of alcohol series with one hour in each series, and two changes in absolute alcohol. The dehydrated lenses were then cleared in cedar wood oil (1 hour), or put in xylene for half an hour. The lenses were then placed in xylene-molten paraffin wax mixture, and incubated at 58°C for one hour. After incubation, the lenses were transferred to pure molten paraffin wax at 58°C for another one hour. Finally, the lenses were embedded in fresh molten paraffin wax. Blocks were made, and sections were cut not more than 5-8 microns thick in a microtome. The sections were fixed on slides, and stained.

Staining Method (Haemotoxylin-Eosin)

1) Sections were dewaxed in xylene for five minutes.
2) Hydrated to water through descending grades of alcohol series (100-50%) with two minutes intervals in each series.
3) The sections were stained with Ehrlich’s haematoxylin for 20-30 minutes.
4) Placed the slides in tap water until the sections were blue in colour (5 minutes).
5) Differentiated in acid-alcohol with a quick dip of slides into the solution.
6) Again placed in tap water until the sections were blue in colour.
7) The sections were counterstained with eosin for three to five minutes.
8) Washed in tap water for three to five minutes.

9) The sections were dehydrated in ascending grades of alcohol series (50-100%) with five minutes intervals in each series.

10) Cleared slowly in alcohol and xylene (3:1, 1:1) with two minutes each.

11) Cleared completely in xylene for one minute.

12) Mounted in DPX (Nice).

Now the slides were ready for viewing under light microscope.

Microphotographs were taken using a Topcon RE200 camera (Japan) attached to the (Olympus) microscope. Fresh extracted whole lens samples of various experimental stages were also photographed by placing them on a slide under the microscope, and by appropriate light illuminations using a 12 V light source.

External photographs of each experimental stages, and their controls, were taken with the aid of macro lenses (52 mm. x 2) attached to a Canon EOS 650 (50 mm., 1:1.8) camera.

**Scanning Electron Microscopy**

**Materials Needed**

1) Glutaraldehyde (3%) (Loba): Glutaraldehyde is normally supplied as 25% or 50% solution. To prepare 100 ml. of 3% glutaraldehyde in 0.1M phosphate buffer, 6 ml. of 50% glutaraldehyde (or 12 ml of 25%) solution was made up to 50 ml with double-distilled water; and added 50 ml. of 0.2 M phosphate buffer, and stored at 4°C.
2) 0.2 M phosphate buffer:

Sodium dihydrogen phosphate \((\text{NaH}_2\text{PO}_4)\cdot\text{H}_2\text{O}\) (Merck) - 641 mg.
Disodiumhydrogen phosphate \((\text{Na}_2\text{HPO}_4\cdot\text{7H}_2\text{O})\) (Merck) - 4.13 g.
Double-distilled water was added to make 100 ml of 0.2M phosphate buffer.

3) Dry acetone (Qualigens): Dry acetone was prepared by adding sufficient amount of copper sulphate to absolute acetone; shaken well, and stored. It was filtered before use.

Double-distilled water was used for preparing graded acetone series from the dry acetone (30, 50, 70, 80, 90 and 95% acetone).

**Procedure**

The lenses were dissected out from the experimental and control rats by the posterior approach, and immediately fixed in three per cent glutaraldehyde in 0.1 M phosphate buffer at 4°C for 24 hrs. After fixation, the specimens were thoroughly washed in 0.1 M phosphate buffer, and kept in the buffer until further process was done. The specimens were trimmed to orient them. The washed sections were then dehydrated in graded acetone series starting from 30% to 100% with 20 minutes in each series, and two changes of 15 minutes each in dry acetone.

The dehydrated specimens underwent critical point drying in liquid \(\text{CO}_2\) in a polaron E 3000 apparatus for an hour. The lenses were then fractured pole-to-pole with a razor blade (Harding *et al.*, 1976), and mounted on aluminium stub with conducting paint, and stored in a desicator until they were taken for sputter coating with conducting metal, gold.
The metal (gold)-coating of the specimens was carried out in vacuum, in an inert atmosphere using argon gas. A coating of uniform thickness was obtained by evaporating gold on to the specimens, kept at a specified distance (cathode specimen distance - 30 mm) for one minute at a current of 21.5 mA and pressure - 0.05 m bar. Using gold, a coating of about 35 nm (350 Å) thickness could be gained under the above conditions in a Balzers SCD 020 sputter device. The gold coated specimens were now ready for observation under scanning electron microscopy (SEM), and stored in a desiccatior until used for SEM observation. The specimens were scanned under a Philips scanning electron microscope 501(B) at 50 kV, and suitable photographs were taken.

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**Part III**

**Biochemical Studies**

The induction and various stages of selenite cataract were produced in both sexes of Wistar rat pups using multiple low (chronic) dosages of 14.4, 19.2, 23, and 27.8 μg. sodium selenite/≈20 g. rat pups subcutaneously, and single high (acute) dosages of 32, 40, 56 and 64 μg./≈20 g. rat pups subcutaneously. For chronic dosages, five doses were given starting on day eight post partum and for acute dosages, a single dose was given on 10th day post partum. In the case of selenite + vitamin-E administered rats, a 2.50 mg. dosage of vitamin-E was administered (5 doses) starting on day 12 post partum subcutaneously along with a 32 μg. sodium selenite dosage/≈25 g. rat pup. The biochemical tests were conducted after the final dose of vitamin-E. In this case, selenite treated rat pups served as the control.
For all other cases of low and high dosages, the vehicle treated rat pups of the same age served as controls.

Biochemical studies were carried out in different experimental groups, and their controls. Nuclear stage of cataract produced by low dosages, nuclear, mature and hyper mature stages of cataract produced by high dosages, along with their age-related controls, vitamin-E + selenite treated cataractous and non-cataractous lenses along with their age-related controls were used for the biochemical study. Besides this, a first to fifth day biochemical analysis of the lens with their controls, (on each day) after the administration of 40 µg. sodium selenite/rat pup, was also carried out. At the required time, the rats were sacrificed and the complete eye lenses were dissected out immediately by the posterior approach. The weight of the lens samples were taken on a balance sensitive to 0.01 mg. The blood was collected wherever necessary, from the jugular vein into a tube containing anticoagulant EDTA, within two minutes after sacrificing the rats. The aqueous humour samples were collected wherever necessary by the method of Kinsey et al. (1963) using a micro syringe.

**Estimation of Protein**

Total, soluble and insoluble lens proteins, aqueous humour and blood proteins were estimated by the method of Lowry et al. (1951).

**Reagents and Chemicals**

1) 0.5N sodium hydroxide (Nice) – Two g. of NaOH was dissolved in 100 ml. distilled water.

2) Sodium carbonate (BDH) solution – 10 g. of Na₂CO₃ was dissolved in 100 ml. of 0.5 N NaOH.
3. Copper sulphate (BDH) (1%) solution – One g. CuSO₄·5H₂O was dissolved in 100 ml distilled water.

4. Potassium tartarate (BDH) (2%) solution – Two g. of potassium tartarate was dissolved in 100 ml. distilled water (The reagents 1-4 may be stored indefinitely).

5. Alkaline copper tartarate solution – 15 ml. reagent 2, 0.75 ml. reagent 3 and 0.75 ml. reagent 4 were mixed thoroughly in a 50 ml. Erlenmeyer flask.

6. Folin-phenol (Loba) reagent – Five ml. of 2N Folin-phenol (commercially available) and 50 ml. of distilled water were mixed thoroughly; it was prepared fresh each time just before the sample estimation.

7. Trichloroacetic acid (Qualigens) (5%) – Five g. of TCA was dissolved in 100 ml. distilled water.


**Procedure**

The weighed lens samples were homogenised in a five volume of 1:1 mixture of TCA (5%) and distilled water. The known volume of blood was suspended in TCA-distilled water mixture. The samples were centrifuged for 10 minutes at 6000 g. The supernatant was discarded, and the pellets were dissolved in one ml. of 0.3 N NaOH which were taken as aliquots for the estimation of total proteins. For the preparation of soluble and insoluble lens protein samples, known weight of lenses were homogenised in a known volume of cold double-distilled water at 4°C, and centrifuged at 6000 g. for about 30 minutes. The supernatants were taken as soluble protein extracts for
estimation. The pellets were dissolved in 1 ml. of 0.3 N NaOH, and taken as insoluble protein extract for estimation of insoluble proteins.

The aqueous humour samples, as such, or diluted with double-distilled water, were taken for the estimation of total proteins.

Test samples (0.1 ml.) were taken; and, to it, distilled water (0.9 ml.) and 1 ml. of alkaline copper tartarate solution, were added. The contents of the tubes were mixed thoroughly, and kept for incubation at room temperature, for 15 minutes. To each incubated sample tubes, three ml. of Folin-Ciocalteu phenol reagent was added, and shaken. The addition to, and mixing of, each tube were completed before preceding to the next. Again the sample tubes were incubated at room temperature, for 45 minutes. The optical density of the resulting blue colour was read at 540 nm on a systronic spectrophotometer. A blank was run along with test samples in which the samples were replaced by 0.3 N NaOH.

The protein content in each sample was calculated using the standard curve obtained by using known quantities of bovine serum albumin. The amount of protein in the sample was calculated directly from the standard curve, and expressed as mg. proteins/mg. lens wet tissue wt. and mg./ml. blood and aqueous humour.

**Estimation of Glutathione**

Determination of glutathione concentration in the samples was carried out by the method of Grunert and Philips (1951).
Reagents and Chemicals

1) Metaphosphoric acid (Loba) (3%) solution – Three g. of metaphosphoric acid was dissolved in 100 ml. distilled water.

2) 0.067M sodium nitroprusside (Nice) solution – 1.996 g. of sodium nitroprusside was dissolved in 100 ml. distilled water.

3) 1.5 M sodium carbonate ((BDH) solution – 12.6 g. of Na₂CO₃ was dissolved in 100 ml. distilled water.

4) 0.067 M sodium cyanide (M and BR) solution – 328 mg. NaCN was dissolved in 100 ml. distilled water.

5) Sodium chloride (BDH) – granular

6) Reduced glutathione (Loba).

Procedure

A known weight of lens samples was homogenised in three ml. of metaphosphoric acid (3%) and one ml. of double-distilled water. The blood samples (0.5 ml.) were haemolysed in one ml. of double-distilled water and 2.5 ml. of metaphosphoric acid (3%). The homogenised samples were saturated with sodium chloride granules. It was mixed well, and centrifuged at 5000 g., for 15 minutes, or allowed to settle slowly in a refrigerator. All procedures were carried out at 4-8°C.

Two ml. of the supernatant was collected. After equilibration at 20°C for 5-10 minutes, one ml. of sodium nitroprusside solution was added, followed immediately by one ml. of sodium carbonate-sodium cyanide (1:1) solution. The optical density of the resulting colour was measured soon after the addition of sodium carbonate-sodium cyanide solution at 520 nm on a
systronic spectrophotometer. Two ml. of metaphosphoric acid (3%) saturated with sodium chloride was used as the blank.

The glutathione concentration in each sample was calculated using reduced glutathione as the standard. The standardised conversion factor was \( \cot \theta = 0.3. \text{Eq.} \ F = 1111.14 \).

**Estimation of Sulfhydryl Groups**

Total, protein bound and non-protein sulfhydryl groups in the lens were carried out by the method of Sedlak and Lindsey (1968).

**Reagents and Chemicals**

1) Ellman’s reagent (0.01M dithiobis nitrobenzoic acid, DTNB) (Sigma) - 396 mg. DTNB was dissolved in 100 ml. sodium citrate (1%).

2) Sodium citrate (Qualigens) (1%) - 1 g. sodium citrate was dissolved in 100 ml. distilled water.

3) 0.02 M ethylene diamine tetra acetic acid - disodium - (EDTA.Na₂) (Nice) - 744 mg. EDTA was dissolved in 100 ml. distilled water.

4) 0.2 M tris (Loba) buffer (pH 8.2) - 2.422 g. tris was dissolved in 100 ml. distilled water.

5) 0.4 M tris buffer (pH 8.9) - 4.845 g. tris was dissolved in 100 ml. distilled water.

6) 50% trichloroacetic acid (TCA) - 50 g. TCA was dissolved in 100 ml. distilled water.

7) Absolute methanol (Qualigens).

8) Reduced glutathione.
Procedure

The weighed lens samples were homogenised in 8 ml. of 0.02 M EDTA at 4°C. The homogenates were kept in the ice bath until used. All buffers and solutions were gassed two to three minutes, with vigorous stream of nitrogen, prior to their use in the procedure.

Estimation of Total Sulphhydryl Groups (TSH)

A 0.5 ml. of the lens homogenate was mixed in 15 ml. test tubes with 1.5 ml. of 0.2 M tris buffer (pH 8.2), and 0.1 ml. of 0.01 M DTNB. The mixture was made up to 10 ml. with 7.9 ml. of absolute methanol. The test tubes were stoppered, and allowed to stand, with occasional shaking, for 15 minutes. Then the tubes were centrifuged at 3000 g. at room temperature for 15 minutes. The absorbance of the supernatant was read at 412 nm in a systronic spectrophotometer. A reagent blank and a sample blank were prepared in the similar manner.

Estimation of Non-Protein Sulphhydryl Groups (NPSH)

Five ml. of the lens homogenate was mixed in a 15 ml. test tube with four ml. of distilled water, and one ml. of 50% TCA. The tubes were shaken at intervals, for 15 minutes, and centrifuged for 15 minutes at 3000 g. Two ml. of the supernatant were mixed with four ml. of 0.4 M tris buffer (pH 8.9), and 0.1 ml. of 0.01 M DTNB. The samples were shaken well; and the optical density was read within five minutes, of the addition of DTNB, at 412 nm against a reagent blank.
The amount of sulfhydryl groups in each sample was calculated, using the standard conversion factor (\( \text{Cot } \theta = 0, \text{ Eq. } F = 1000 \)), using reduced glutathione as the standard.

**Determination of Protein Bound Sulfhydryl Groups (PSH)**

Protein bound sulfhydryl groups were determined by subtracting the NPSH from the TSH.

**Estimation of Ascorbic Acid**

The estimation of ascorbic acid in the lens was carried out by the method of Roe and Kuther (1943).

**Reagents and Chemicals**

1) 2,4 dinitrophenyl hydrazine (DNPH) (Nice) (2%) solution - Two g. DNPH was dissolved in 100 ml. of 9 N \( \text{H}_2\text{SO}_4 \)

2) \( \text{H}_2\text{SO}_4 \) (BDH) (9 N) - 26.19 ml. conc. \( \text{H}_2\text{SO}_4 \) was diluted with 73.81 ml. distilled water.

3) 85% \( \text{H}_2\text{SO}_4 \) - 85 ml. conc. \( \text{H}_2\text{SO}_4 \) was diluted to 100 ml. with distilled water.

4) 10% thiourea (Nice) - 10 g. thiourea was dissolved in 100 ml. of 50% alcohol.

5) TCA (6%) - 6 g. TCA was dissolved in 100 ml. of distilled water.

6) TCA (4%) - 4 g. TCA was dissolved in 100 ml. of distilled water.

7) Norit reagent - Two g. activated charcoal (BDH) was put in 100 ml. of TCA (6%), shaken well, and kept for 10-15 minutes. Filtered through Whatman paper No. 42, and used. It was always freshly prepared.
8) Standard ascorbic acid (CDH) solution – 50 mg. of ascorbic acid was dissolved in 50 ml. of TCA (6%). One ml. of this solution was diluted to 100 ml. with TCA (4%), so that one ml. contains 10 µg. ascorbic acid.

Procedure

The weighed lenses were homogenised in five ml. of Norit reagent. One ml. of blood was diluted with Norit reagent. To two ml. of diluted sample or homogenate, 0.5 ml. of 2,4, DNPH was added followed by a drop of 10% thiourea. The sample tubes were kept in a boiling water bath for 15 minutes; then cooled in ice for 15 minutes. After cooling, 2.5 ml. of 85% H₂SO₄ was added to each tube, and the tubes were left for 30 minutes. The absorbance of the sample solution was read at 540 nm in a systronic spectrophotometer. The standard tube was run with four ml. of ascorbic acid solution (10 µg./ml.), and the blank tube with four ml. of TCA (6%) instead of tissue homogenate. The ascorbic acid content in each sample was calculated, using the standard concentration and O.D., and expressed in µg./100 g. wet lens tissue wt. or µg./ml. of blood.

Estimation of Nucleic Acids

The extraction of deoxy ribonucleic acid (DNA) and ribonucleic acid (RNA) from the lens samples was carried out by the method of McMaster and Modak (1977).

Reagents and Chemicals

1) Trichloroacetic acid (TCA) (8%) – Eight g. TCA was dissolved in 100 ml. of acetone.
2) 0.25M perchloric acid (PCA) (Merck) – 2.512 ml. was diluted in 97.488 ml. of distilled water.

3) 0.5M perchloric acid (PCA) – 5.023 ml. was diluted in 97.977 ml. of distilled water.

4) Perchloric acid (PCA)(1M) – 10.046 ml. was diluted in 89.954 ml. of distilled water.

5) 0.9M sodium hydroxide – 3.6 g. NaOH was dissolved in 100 ml. of distilled water.

6) Orcinol (Loba) reagent – 200 mg. of orcinol was dissolved in 70 ml. of concentrated hydrochloric acid, and 10 ml. of 0.004 M copper chloride; and made up to 100 ml. with con. HCl (BDH); and stored at 4°C in a dark bottle.

7) 0.004 M copper chloride (CDH) – 68 mg. CuCl2.H2O was dissolved in 100 ml. conc. HCl.

8) Diphenylamine (Loba) reagent – 1.5 g. diphenylamine was dissolved in 1.5 ml. of concentrated sulphuric acid, and made up to 100 ml. with glacial acetic acid. Just before use, 0.1 ml. of acetaldehyde (Merck) (16 mg./ml.) was added to 20 ml. diphenylamine reagent.

9) Iso amyl alcohol (Qualigens)

10) Deoxy ribonucleic acid (DNA) (Himedia)

11) Ribonucleic acid (RNA) (Sigma)

**Procedure**

A known weight of lens samples was homogenised in five ml. of 1:1 mixture of TCA (8%) in acetone and distilled water. Samples were left on ice for 15 minutes; and centrifuged at 4000 g. for 10 minutes at 2°C. The
supernatant was discarded, and the above step was repeated. The resulting pellet was resuspended in five ml. of 0.25 M PCA, left on ice for 1.5 minutes, and centrifuged for 10 minutes of 4000 g. at 2°C. The supernatant was discarded, and the above step was repeated. The pellet was hydrolysed in three ml. of 0.3 M NaOH for one hour at 37°C, left on ice for 15 minutes, precipitated with one ml. of PCA (1M) kept on ice for one hour, and centrifuged at 4000 g. for 10 minutes at 2°C. The supernatant was used for the determination of RNA while the pellet was further processed, in order to estimate DNA.

The pellet was hydrolysed in two ml. of 0.5 M PCA for 15 minutes at 70°C, cooled on ice for 15 minutes, and centrifuged at 4000 g. for 10 minutes at 2°C. The supernatant was used for the determination of DNA.

**Estimation of Ribonucleic Acid (RNA)**

RNA content of the extracted supernatant was carried out by the method of Ceriotti (1955), using orcinol reagent.

One ml. of the RNA extracted supernatant was mixed with 1.5 ml. of orcinol reagent in a 10 ml. test tube. The sample tubes were incubated for 40 minutes in a boiling water bath. The tubes were chilled under running tap water for 30 minutes. Then added 5 ml. of iso-amyl alcohol to extract the colour. The tubes were mixed thoroughly, and centrifuged for one minute at 4000 g. The optical density was read at 675 nm in a systronic spectrophotometer. A sample blank was prepared as above.

The concentration of RNA in each sample was calculated, using the standardised regression formula \( x = 189.65, \ y = 79.74 \)
Estimation of Deoxyribonucleic Acid (DNA)

The estimation of DNA contents in the extracted supernatant was made by method of Giles and Myers (1965), using diphenyl amine reagent.

To one ml. of DNA extracted supernatant, taken in a 10 ml. test tube, two ml. of diphenyl amine reagent was added. The contents were mixed thoroughly, and incubated at 37°C for 16 hours at 37°C. The incubation time was kept constant all through. After incubation, the optical density of the resulting blue colour was read at 620 nm in a systronic spectrophotometer against blank. A sample blank was prepared using 0.5 M PCA.

The concentration of DNA in each sample was calculated, using the standardised regression formula \( x = 371.89, \ y = 1.19 \)

Estimation of Glucose

The amount of glucose content in the lens was estimated by the method of Nelson (1944) and Somogyi (1945).

Reagents and Chemicals

1) Zinc sulphate (Nice) (5%) solution – Five g. \( \text{ZnSO}_4\cdot7\text{H}_2\text{O} \) was dissolved in 100 ml. distilled water.

2) 0.5 N sodium hydroxide solution – Two g. \( \text{NaOH} \) was dissolved in 100 ml. distilled water.

3) Alkaline copper tartarate solution.

Solution A

12.5 g. of anhydrous sodium carbonate, 12.5 g. of potassium sodium tartrate (Rochelle salt), 10 g. of sodium bicarbonate (CDH), and 100 g. of anhydrous sodium sulphate (Merck) were dissolved in about 400 ml. of
boiling distilled water, and boiled for eight to ten minutes more, cooled, and made up to 500 ml; kept for three to four days, filtered if necessary, and stored at room temperature.

**Solution B**

15 g. of CuSO₄.5H₂O was dissolved in 80 ml. of boiling water, cooled, added two drops of concentrated sulphuric acid, and made up to 100 ml.

One ml. of solution B was mixed with 24 ml. of solution A on the day of the experiment, filtered before use, and discarded the mixture after 24 hours.

4) **Arsenomolybdate reagent** – Five g. of ammonium molybdate (Nice) was dissolved in 90 ml. of distilled water, and 4.2 ml. of concentrated H₂SO₄ was added to it. Mixed and then added with stirring, a solution of 600 mg. of disodium hydrogen arsenate (Na₂HAsO₄, 7H₂O) (CDH) in five ml. of distilled water, and made up to 100 ml., placed at 37°C for 48 hrs. Stored in a glass stoppered brown bottle.

**Procedure**

The weighed lens samples were homogenised in two ml. of alkaline copper tartarate solution, and two ml. distilled water. The blank samples of known volume were diluted with two ml. of alkaline tartarate solution.

The homogenates and diluted samples were taken in 10 ml. test tubes, and added 0.2 ml. of sodium hydroxide solution (0.5 N) and 0.2 ml. of zinc sulphate solution (5%). All tubes were centrifuged for three to five minutes. 0.5 ml. of the supernatants were added to one ml. of alkaline copper tartarate solution, and boiled for 15 minutes. 0.5 ml. distilled water was used as the
blank. After cooling, one ml. of arsenomolybdate reagent was added to all the tubes: diluted the test mixtures with 7.5 ml. of distilled water: and the optical density was read at 540 nm. The concentration of glucose in each sample was calculated, using the standard glucose solution, and the standardised conversion factor (cot θ = 0.80; Eq. F = 156.25).

**Estimation of Fructose**

Fructose estimation was carried out by the method of Foreman *et al.* (1973).

**Reagents and Chemicals**

1) 0.1% resorcinol (Nice) – 100 mg. resorcinol was dissolved in 100 ml. distilled water.
2) Perchloric acid (5%) – 3.5 ml. 70% perchloric acid was made up to 100 ml. with distilled water.
3) 30% hydrochloric acid (BDH) – 30 ml. concentrated HCl was made up to 100 ml. with distilled water.
4) 95% ethyl alcohol – 95 ml. absolute alcohol was made up to 100 ml. with distilled water.
5) D-fructose (Loba).

**Procedure**

The weighed lens samples were homogenised in five ml. of perchloric acid (5%). Two ml. of the lens homogenates were taken in a 10 ml. test tube. One ml. of 0.1% resorcinol, and three ml. of 30% hydrochloric acid were added to all the tubes. The sample tubes were incubated in a water bath at
80°C for one hour. After incubation, all the tubes were cooled and the optical density measured at 410 nm. Two ml. of PCA (5%) was taken as the blank.

The concentration of fructose in each sample was calculated using standardised conversion factor (cot θ = 0.90; Eq. F = 123.46).

**Estimation of Thiobarbituric Acid (TBA) Reactive Material or Malondialdehyde (MDA)**

The MDA concentration was assayed by the method of Niehans and Samuelson (1968).

**Reagents and Chemicals**

1) TCA-TBA-HCl reagent – 15 g. trichloroacetic acid (TCA) and 375 mg. thiobarbituric acid (TBA) (CDH) were dissolved in 100 ml. of 0.25 N HCl. The solution was mildly heated to assist in the dissolution of TBA.

2) 0.25N Hydrochloric acid (HCl) – 2.14 ml. of concentrated HCl was made up to 100 ml. in distilled water.

3) 0.025 M Tris-HCl (pH 7.5) – 90.6 mg. tris was dissolved in 30 ml. distilled water and pH adjusted to 7.4 with 0.1 N HCl (about 60 drops).

4) 0.1 N HCl – 0.42 ml. con. HCl was made up to 50 ml. with distilled water.

**Procedure**

The weighed lens samples were homogenised in ice cold 0.025 M tris-HCl. One ml. of the lens homogenate was taken in a test tube, and two ml. of TCA-TBA-HCl reagent were added, and mixed thoroughly. The sample tubes were heated for 15 minutes in a boiling water bath. After cooling the
floculent precipitate was removed by centrifugation at 1000 g. for 10 minutes. The absorbance was measured at 535 nm against a blank that contained all the reagent minus the lens homogenate, which was replaced by distilled water. The MDA concentration of the samples was calculated, using an extinction coefficient of $1.56 \times 10^5 \text{m}^{-1}\text{cm}^{-1}$. The MDA concentration was expressed as mM/100 g. tissue wt.

**Elemental Analysis**

The analysis of potassium and sodium was done using Flame photometer; calcium and magnesium were analysed by atomic absorption spectrophotometer, with reference to Duncan and Bushell (1975) method.

The experimental and control Wistar rat lenses were extracted by the posterior approach, and blotted on filter paper, moistened with deionized water, and ethylene glycol-bis-(β-amino ethyl ether) N,N'-tetra acetic acid (EGTA) (1 mM). The blotted and weighed lenses were further processed for analysing potassium, sodium, calcium and magnesium. The concentration of these elements was expressed in percentage.

**Determination of Potassium and Sodium**

The concentration of potassium and sodium in the lens samples was determined by Flame Photometry.

**Reagents and Chemicals**

1) Standard solutions:
   a. Potassium stock solution (1000 ppm) - 1.9068 g. of AR grade potassium chloride (KCl) was dissolved in 1000 ml. of distilled water.
b. Sodium stock solution (1000 ppm).

c. Potassium and sodium working standard solution - 50 ml. of 1000 ppm potassium stock solution was diluted to 500 ml. (100 ppm). Pipetted five, 10, 15 and 20 ml. portions of 100 ppm stock solution to 100 ml. standard flask and made upto mark with distilled water. The concentration of potassium in the solutions were five ppm, 10 ppm, 15 ppm and 20 ppm. Similarly, sodium working standard was made from 1000 ppm sodium stock solution.

2) Lanthanum chloride (Sigma)
3) Nitric acid (HNO₃) (Merck)
4) Hydrochloric acid (HCl₂)
5) Hydrogen peroxide (H₂O₂) (Merck)

**Procedure**

The weighed lens samples were digested to dryness alternately in three ml. concentrated HNO₃, and, then, in one ml. H₂O₂. Residual salt (ash) was dissolved in HCl (1%) containing 0.2% lanthanum chloride, and made up to 50 ml. This solution was used for potassium (K) and sodium (Na) analysis using flame photometry.

The Flame Photometer was set to measure either potassium or sodium. Aspirated the appropriate working standards, and noted the readings. Constructed separate standard graphs for potassium and sodium relating meter readings to the concentration of standards.

Now sprayed the sample test solutions, and the readings were noted. The concentration of potassium or sodium was calculated from the standard graphs.
Determination of Calcium and Magnesium

The concentration of calcium and magnesium in the lens samples was analysed using Atomic Absorption Spectrophotometer.

Reagents and Chemicals

1) Compensating solution for calcium:
   a. Preparation of stock solutions (1000 ppm. phosphorous + 5000 ppm. potassium and 1000 ppm. magnesium)
      4.3928 g. of potassium dihydrogen phosphate (KH₂PO₄, dried at 40°C) and 7.1266 g. potassium chloride (KCl, dried at 105°C for two hours) were dissolved in distilled water, and made upto 1000 ml.
      10.1308 g. magnesium sulphate (MgSO₄·7H₂O) was dissolved in distilled water, and made up to 1000 ml. to get 1000 ppm. magnesium.
   b. Preparation of compensating solution – Five ml. of phosphorous + potassium stock solution, and six ml. of 1000 ppm. magnesium stock solution were pipetted and made upto 250 ml. with distilled water.

2) Standard Calcium solution
   a. Primary standard–1000 ppm – 2.4970 g. of calcium carbonate (CaCO₃ dried for two hours at 105°C) was weighed into a 250 ml. conical flask. The flask was covered with a small funnel, and slowly added 50 ml. of hydrochloric acid (1 N). The solution was boiled to expel carbon dioxide. Cooled, and made up to 1000 ml. with distilled water.
b. Secondary standard - 50 ppm – 50 ml. of the 1000 ppm. calcium solution was pipetted into a one litre flask and made up to the mark.

c. Working standards - 1-10 ppm – Four, eight, 16, 24, 32, and 40 ml. of the 50 ppm. calcium solution was pipetted into 200 ml. volumetric flask. To each flask 10 ml. of 0.2% lanthanum chloride solution, and 10 ml. of compensating solution for calcium were added. The solution was made up to the mark with water. The resulting solutions gave one, two, four, six, eight and 10 ppm., calcium working standards. A blank was prepared using lanthanum chloride and the compensating solution.

3) Compensating solution for magnesium

a. Preparation of stock solutions (1000 ppm. phosphorous + 1000 ppm. potassium and 1000 ppm. calcium)

4.3928 g. of \( \text{KH}_2\text{PO}_4 \) (dried at 40°C) and 7.1266 g. of \( \text{KCl} \) (dried at 105°C for two hours) were dissolved in distilled water and made up to 1000 ml.

2.4970 g. of calcium carbonate (dried for two hours at 105°C) was weighed into a conical flask. The flask was covered with a small funnel and slowly added 50 ml. of hydrochloric acid (1 N). The solution was boiled to expel carbon dioxide. Cooled, and made up to 1000 ml. with distilled water to get 1000 ppm. calcium.

b. Preparation of compensating solution – One ml. of phosphorous and potassium stock solution and 12.5 ml. of calcium stock
solution were pipetted and made up to 250 ml. with distilled water.

4) Magnesium standard solutions

a. Primary standard - 1000 ppm - 10.1308 g. of pure magnesium sulphate was dissolved in distilled water, and made up to 1000 ml.

b. Secondary standard - 100 ppm - 50 ml. of the 1000 ppm. stock solution was pipetted and made up to 500 ml. with distilled water.

c. Tertiary standard - five ppm - 25 ml. of the 100 ppm. stock solution was pipetted and made up to 500 ml. with distilled water.

d. Working standards 0.1-1 ppm - Four, six, eight, 16, 24, 32, and 40 ml. of 5 ppm magnesium solution were pipetted into 200 ml. volumetric flask. To each flask 10 ml. of 0.2% lanthanum chloride solution and 10 ml. compensating solution for magnesium were added. Made up to the mark with distilled water. The resulting solutions gave 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 and 1.0 ppm. magnesium working standards. A blank was prepared using lanthanum chloride and the compensating solution.

5) Lanthanum chloride

6) Nitric acid (HNO₃)

7) Hydrochloric acid (HCl)

8) Hydrogen peroxide (H₂O₂)
Procedure

The weighed lens samples were digested to dryness alternatively in three ml. concentrated HNO₃, and, then, in one ml. H₂O₂. Residual salt (ash) was dissolved in HCl (1%) containing 0.2% lanthanum chloride, and made upto 50 ml. This solution was used for calcium (Ca) or magnesium (Mg) analysis. using the GBC Avanta (Australia) atomic absorption spectrophotometer.

The atomic absorption spectrophotometer was set to measure either calcium or magnesium. Aspirated the appropriate working standards, and noted the readings at 422.7 nm for calcium, using calcium hollow cathode lamp, or at 285.2 nm for magnesium, using magnesium hollow cathode lamp. Constructed separate graphs for calcium and magnesium, relating meter readings to the concentration of standards.

Now sprayed the sample test solutions, and the readings were noted. The concentration of calcium or magnesium was calculated from the standard graph.

Assay of Adenosine Triphosphatase (ATPase) (E.C. 3.6.1.3) Activity

The ATPase activity in the lenses was assayed by the method of Quinn and White (1968).

Reagents and Chemicals

1) ATP (Sigma) (3 mM) solution - 82.673 mg. ATP disodium salt was dissolved in 50 ml. distilled water, and stored at 4°C.

2) Tris-HCl (pH 7.4).
Solution A

Tris-Hydroxymethyl amino methane (Loba) - 2.42 g. tris was dissolved in 100 ml. distilled water.

Solution B

Con. hydrochloric acid (HCl) - 8.5 ml. HCl was made up to 100 ml. with distilled water.

25 ml. of solution A was mixed with 42.5 ml. of solution B. Measured the pH and, if needed, adjusted with solution B; made up the solution to 100 ml. with distilled water.

3) 250 mM sucrose (Qualigens) solution buffered with Tris-HCl (pH 7.4) - 115 g. sucrose was dissolved in 50 ml. Tris-HCl.

4) 150 mM sodium chloride - 877 mg. NaCl₂ was dissolved in 100 ml. distilled water.

5) 30 mM potassium chloride (Nice) - 224 mg. KCl₂ was dissolved in 100 ml. distilled water.

6) Magnesium chloride (Qualigens) (3 mM) - 60.993 mg. MgCl₂ was dissolved in 100 ml. distilled water.

7) 10% trichloro acetic acid (TCA) - 10 g. TCA was dissolved in 100 ml. distilled water.

Procedure

The weighed lens samples were homogenised in one ml. of double-distilled water at 4°C. In a 10 ml. test tube 0.3 ml. of ATP (substrate) solution was taken, and, to it, 0.1 ml. sodium chloride, 0.1 ml. potassium chloride, 0.1 ml. magnesium chloride, 0.1 ml. of lens homogenate, and 0.2 ml. tris buffered sucrose solution, were added. The contents in the tubes were
mixed well, and incubated at 30°C for 30 minutes, with intermittent shaking. At the end of the incubation period, 0.2 ml. of cold TCA solution was added. The mixture was kept for 10 minutes at 4°C for complete precipitation. The solution was centrifuged, and the supernatant was collected.

The ATPase activity was measured in terms of the inorganic phosphate formed during the reaction.

**Determination of Inorganic Phosphate**

The inorganic phosphate was measured in the supernatant by the method of Fiske and Subha Row (1925).

**Reagents and Chemicals**

1) 2.5% acidic ammonium molybdate solution (Nice) – 2.5 g. ammonium molybdate was dissolved in 20 ml. distilled water, and 50 ml. of 10 N sulphuric acid was added to it. Mixed well, and made up to 100 ml. with distilled water.

2) Sulphuric acid (H₂SO₄) (10 N) – 29.1 ml. concentrated H₂SO₄ was made up to 100 ml. with distilled water.

3) Aminonaphthol sulphonic acid reagent (ANSA) (Loba) – 200 mg. ANSA, and 1.2 g. sodium sulphite, were dissolved in 56 ml. distilled water.

4) Standard solution of phosphate – 177 mg. of potassium dihydrogen phosphate (KH₂PO₄) (Qualigens) was dissolved in 100 ml. distilled water (1.77 mg./ml.).

For a working standard solution, 7.5 ml. of the above solution was made up to 50 ml. with distilled water. The concentration of KH₂PO₄ in one ml. of this working standard was 266.5 μg.
Procedure

One ml. of the supernatant was taken, and 0.2 ml of acidic ammonium molybdate, 0.7 ml of distilled water, and 0.1 ml ANSA reagent, were added. The contents were thoroughly mixed, and kept standing for one hour at room temperature for the colour development. The samples and blanks were run simultaneously. The resulting greenish blue colour was read at 620 nm.

The activity of ATPase was expressed in terms of the amount of the inorganic phosphate formed per hour/mg. tissue weight.

Assay of Acid Phosphatase (ACP) (E.C.3.1.3.2)

Acid phosphatase was assayed by the method of Bessey et al. (1946).

Reagents and Chemicals

1) 0.5 M citrate buffer (pH 4.8)

Solution A

Sodium citrate - 14.705 g. was dissolved in 100 ml. distilled water.

Solution B

Citric acid - 10.507 g. was dissolved in 100 ml. distilled water.

27 ml. of solution A was mixed with 23 ml. of solution B, adjusted the pH to 4.8 and made up to 100 ml. with distilled water.

2) 4-nitrophenyl phosphate solution (Merck) (substrate buffer) - 165 mg.

4-nitrophenyl phosphate was dissolved in 100 ml. of 0.5 M citrate buffer.

3) 0.1 N sodium hydroxide (NaOH) - 400 mg. NaOH was dissolved in 100 ml. distilled water.
Procedure

The weighed lens samples were homogenised in five ml. of double-distilled water. 0.6 ml. 4-nitrophenyl phosphate solution was taken in a 10 ml. test tube, and incubated at 37°C for five minutes. Then 0.2 ml. of the lens homogenates were added, and mixed well. The tubes were incubated at 37°C for 30 minutes in a water bath with constant shaking, and thereafter four ml. of 0.1 N NaOH was added to each sample tube, and thoroughly shaken. The optical density was read at 410 nm.

The activity of acid phosphatase in each sample was calculated, with respect to the quantity of p-nitrophenol liberated, using the p-nitrophenol as standard (Conversion factor = 0.741). The activity was expressed as μ moles of p-nitrophenol liberated 100 mg. fresh tissue weight/30 minutes.

Assay of Glucose-6-Phosphate Dehydrogenase (G-6-PDH) (E.C.1.1.1.49) Activity

The activity of G-6-PDH in the lens was assayed by the method of Ells and Kirkman (1961).

Reagents and Chemicals

1) 0.5 M Tris buffer (pH 7.5) – 6.057 g. tris was dissolved in 100 ml. distilled water.

2) Magnesium chloride (1 M) – 20.331 g. MgCl₂ was dissolved in 100 ml. distilled water.

3) 0.01% 2-6-dichlorophenol indophenol (DCPI) (Loba) – 10 mg. DCPI was dissolved in 100 ml. distilled water.
4) 0.01 M Triphosphopyridine nucleotide (TPN) (Sigma) – 787 mg. TPN (NADP) was dissolved in 100 ml. distilled water.

5) Phenazine methosulphate solution (PMS) (Sigma) – Five mg. PMS was dissolved in 100 ml. distilled water.

6) 0.02 M Glucose-6-phosphate (Loba) solution – 608.2 mg. G-6-P was dissolved in 100 ml. of 0.5 M Tris buffer (pH 7.5).

Procedure

The weighed lens samples were homogenised in one ml. of double-distilled water. The insoluble parts were removed by centrifuging the homogenates at 3000 g. for 30 minutes at 4°C.

To each sample tubes, one ml. of tris buffer, 0.1 ml. of MgCl₂, 0.4 ml. of 2-6-dichlorophenol indophenol, 0.1 ml. of TPN solution, 0.4 ml. of lens homogenate and 0.5 ml. of PMS solution were added. The tubes were allowed to stand for 10 minutes at room temperature. Then 0.5 ml. of G-6-P was added. The total volume in each tube was made to six ml. with distilled water. The reduction of DCPI was measured at 620 nm using water blank adjusted to zero. The readings were taken at 30 seconds intervals for five minutes. The activity of the enzyme was expressed as O. D. units change/minute/mg. lens wet wt.

Assay of Succinic Dehydrogenase (SDH) (E.C.1.3.98.1) Activity

The estimation of SDH was made by the method of Kun and Abood (1949) and Beatty et al. (1966).
Reagents and Chemicals

1) 0.2 M phosphate buffer (pH 7.6)
   A. Disodium hydrogen phosphate (Na$_2$HPO$_4$) – 7.16 g. of Na$_2$HPO$_4$
      in 100 ml. distilled water.
   B. Dihydrogen potassium phosphate (KH$_2$PO$_4$) – 2.72 g. of KH$_2$PO$_4$
      in 100 ml. distilled water.
   80 ml. solution A was mixed with 20 ml. of solution B, adjusted
   pH 7.6.

2) 0.1 M sodium succinate (Loba) – 2.70 g. sodium succinate in 100 ml.
   distilled water.

3) 0.1% Triphenyl tetrazolium chloride (TTC) (BDH) solution – 100 mg.
   TTC was dissolved in 100 ml. distilled water. It was prepared fresh, as
   required.

4) 30% Trichloroacetic acid – 30 g. TCA was dissolved in 100 ml. distilled
   water.

5) Ethyl acetate (Ranbaxy)

Procedure

The weighed lens samples were homogenised in one ml. distilled water,
and diluted to five ml.

The assay mixture consisted of one ml. of sodium succinate, one ml. of
phosphate buffer, one ml. of TTC solution, and 0.4 ml. of the lens
homogenate. After thorough mixing all the samples were incubated at 37°C
for three hrs. The reaction was stopped by adding 30% TCA. A sample blank
and a reagent blank were run simultaneously. The formazan was extracted
into seven ml. of ethyl acetate by vigorous shaking for 30 seconds. All the
tubes were then centrifuged at 1500 g. for five minutes. The optical density of
the supernatant was read at 420 nm.

The activity of succinate dehydrogenase was calculated, using
standardised conversion factor. The enzyme activity was expressed as µg.
formazan formed/hour/mg. lens weight. Regression formulae: \( x = 1.866 + 
522.21y \).

**Assay of γ-Glutamyl Cysteine Synthetase (γ-GCS) (E.C.6.3.2.2)**

The activity of γ-GCS in the lens was carried out by measuring the
inorganic phosphate formed from ATP by the method of Fiske and Subha
Row (1925), using standard γ-GCS assay procedure (Rathbun, 1967).

**Reagents and Chemicals**

1) 0.02 M Tris sulphate (pH 7.6)
   A. Sodium sulphate (\( \text{Na}_2\text{SO}_4 \)) – 284 mg. in 100 ml. of 3 mM EDTA.
   B. Tris – 242 mg. in 100 ml. of EDTA (3 mM)

   55 ml. of solution A was mixed with 100 ml. of solution B. Adjusted
   pH to 7.6

2) Ethylene diamine tetra acetic acid (EDTA) (3 mM) – 116.72 mg. EDTA
   was dissolved in 100 ml. distilled water.

3) 15 mM L-Glutamate (CDH) – 221 mg. L-glutamate was dissolved in
   100 ml. distilled water.

4) L-Cysteine (CDH) (5 mM) – 79 mg. L-cysteine was dissolved in 100 ml.
   distilled water.

5) Disodium ATP (2 mM) – 110 mg. ATP was dissolved in 100 ml.
   distilled water.
6) 50 mM Magnesium sulphate (MgSO₄) (CDH) – 601.85 mg. MgSO₄ was dissolved in 100 ml. distilled water.

7) 10% Trichloro acetic acid (TCA) – 10 g. TCA was dissolved in 100 ml. distilled water.

**Procedure**

The weighed lens samples were homogenised in an ice-cold 0.02 M tris buffer at 4°C in chilled glass homogenising tubes in a cold room. After centrifuging the homogenate at 10,000 g. for 15 minutes, the supernatant was dialysed against the same buffer. The assay mixture consists of 0.1 ml. 15 mM L-glutamate, 0.1 ml. L-cysteine (5 mM), 0.1 ml. disodium ATP (2 mM), 0.1 ml. of 50 mM MgSO₄, 0.1 ml. 100 mM Tris-sulphate, and 0.45 ml. of the lens homogenate. In the control, one of the substrate L-cysteine was omitted. The test and control were incubated for one hour, at 37°C and terminated by the addition of one ml. of 10% TCA. The samples were centrifuged, and the supernatant was used for the estimation of inorganic phosphate, as described earlier.

The activity of the γ-GCS was expressed in terms of the quantity of the inorganic phosphate formed per hour/g. lens weight.

**Assay of Glutathione Reductase (GSH-R) (NADPH: Oxidised Glutathione Oxidoreductase) (E.C.1.6.4.2)**

The GSH-R activity was assayed by the method of Goldberg and Spooner (1983).

**Reagents and Chemicals**

1) 0.12 M phosphate buffer (pH 7.6) – 1.633 g. potassium dihydrogen phosphate (KH₂PO₄) was dissolved in 80 ml. distilled water, the pH
was adjusted to 7.2 with 1M NaOH and diluted to 100 ml. with distilled water.

2) NaOH (1 M) – 4 g. NaOH was dissolved in 100 ml. distilled water.

3) 15 mM Ethylene diamine tetraacetic acid (EDTA) – 56 mg. EDTA was dissolved in 100 ml. distilled water.

4) 9.6 mM reduced nicotinamide-adenine dinucleotide (NADPH) (Sigma) – Eight mg. NADPH, tetra sodium salt was dissolved in one ml. of one per cent sodium dihydrogen carbonate (NaH₂CO₃) solution.

5) Sodium dihydrogen carbonate (1%) solution – One g. NaH₂CO₃ was dissolved in 100 ml. distilled water.

6) 65.3 mM oxidised glutathione (GSSG) (Sigma) – 40 mg. GSSG was dissolved in One ml. distilled water.

**Procedure**

The weighed lens samples were homogenised in 10 parts chilled phosphate buffer (i.e., 2.2 ml. for 220 g. lens tissue) in a glass homogenizer. The homogenate was centrifuged at 4°C in a centrifuge at 12,000 g. for 10 minutes. The supernatant obtained was used for the enzyme assay.

Pipetted into the sample tubes, 2.60 ml. phosphate buffer, 0.01 ml. EDTA, 0.10 ml. GSSG, and 0.10 ml. lens homogenates sequentially, and mixed thoroughly, washed for five minutes, and 0.05 ml. NADPH was added. It was mixed thoroughly, and the absorbance was taken for 10 minutes, with a time interval of one minute at 339 nm.

The GSH-R activity was calculated by using the formulae $4.68 \times 10^3 \times \frac{\Delta A_b}{\Delta t}$ (change in absorbance/minute) where $4.68 \times 10^3$ was the factor used for calculating the GSH-R activity by the assay method optimised.
for any source (phosphate buffer) at a wave length 339 nm. The enzyme activity was expressed as units/mg. wet tissue wt.

**Determination of Water Contents**

The water contents of the lens samples were determined by measuring the weight difference before and after drying at 80-90°C for two hours in an incubator. By subtracting the final weight of the lens samples after drying with that of the initial weight of the lens after the lens extraction gave out the water contents in the lens samples. This was expressed in mg. per lens. From this the percentage of solid matter and water in the lens samples were calculated.

**Statistical Analysis**

The experimental values were statistically analyzed using one way classification of analysis of variance (ANOVA) and ‘t-test’ (Snedcor and Cohran, 1967). P values of <0.01 and <0.05 were considered as statistically significant. The values were considered as highly significant based on the increase observed in the F values for each parameters.

**Part IV**

**Electrophoretic Studies**

Electrophoretic studies on selenite cataract were studied on a specific sodium selenite dosage optimised (40 µg./rat pup). The studies were conducted on selected stages after selenite administration, as described earlier. The experimental and their control lens proteins were run on a 12.5% sodium dodecyl sulphate polyacrylamide mini gel electrophoretic (SDS PAGE) system.
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

Qualitative analysis of the proteins was done using SDS-PAGE as described by Laemmli (1970). The proteins were separated on a mini vertical slab gel (BROVIGA, Madras) electrophoretic apparatus.

Materials Needed

1. Acrylamide-bisacrylamide (30: 0.8, w/w): 30 g. acrylamide (Loba), and 0.8 g. of bisacrylamide (Kemphasol), were dissolved in double-distilled water, and the volume was made up to 100 ml. The solution was then filtered through Whatman No. 1 filter paper, and stored at 4°C.

2. SDS (Loba) (10% w/v): 10 g. of SDS was dissolved, and made up to 100 ml. in double-distilled water.

3. Ammonium persulphate (Nice) solution: 150 mg. of ammonium persulphate was dissolved in one ml. of double-distilled water, and made fresh just before use.

4. TEMED (Loba) (Tetramethyl ethylene diamine).

5. Electrophoresis buffers
   a) Stacking gel buffer: 0.5 M Tris-HCl (pH 6.8) - Six g. of Tris (Loba) was dissolved in 40 ml. of double-distilled water, and pH was adjusted to 6.8 with HCl (BDH), and the final volume was made up to 100 ml.
   b) Resolving gel buffer: 1.5 M Tris-HCl (pH 8.8) - 18.1 g. of Tris was dissolved in 40 ml. of double-distilled water, and the pH was adjusted to 8.8 with HCl, and the final volume was made up to 100 ml.
c) Electrode buffer: Tris-glycine (pH 8.3) - Six g. of Tris, 28.8 g. of glycine (Loba), and one g. of SDS were dissolved in 1000 ml. of double-distilled water.

6. SDS-sample buffer: 0.0625 M Tris-HCl (pH 6.8) with SDS (5%), Mercapto ethanol (2%) (Sigma), 10% glycerol (Merck) and 0.1% bromophenol blue (Loba) as tracking dye was prepared.

7. Phosphate buffered saline (PBS solution) pH 7.4: 0.9 g. NaCl₂, 0.05 g. KCl₂, 0.02 g. KH₂PO₄, and 0.115 g. Na₂HPO₄ were dissolved in 100 ml. of double-distilled water.

8. Molecular weight markers (Std. Sigma Dalton Mark VI for SDS PAGE)
   - Lysozyme: 14,300 KDa
   - β-lactoglobulin: 18,400 KDa
   - Trypsinogen: 24,000 KDa
   - Pepsin: 34,700 KDa
   - Albumin egg: 45,000 KDa
   - Albumin bovine: 66,000 KDa

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**Recipe for the Preparation of SDS-Polyacrylamide Gel**

<table>
<thead>
<tr>
<th>Stock solution (ml.)</th>
<th>Stacking gel (4%)</th>
<th>Final acrylamide concentration of resolving gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7.5%</td>
</tr>
<tr>
<td>Acrylamide bisacrylamide solution</td>
<td>2.5</td>
<td>10.0</td>
</tr>
<tr>
<td>Stacking gel buffer</td>
<td>5.0</td>
<td></td>
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<tr>
<td>Resolving gel buffer</td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Double-distilled water</td>
<td>5.6</td>
<td>17.6</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>Ammonium persulphate solution</td>
<td>0.2</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Gloves would always be used since both acrylamide and bisacrylamide were neurotoxic in salt and solutions. However, polymerised gels were safe to handle.

**Preparation of Slab Gels**

A glass plate sandwich was prepared, using clean glass plates with three teflon spacers of 1.5 mm thickness, and sealed by coating with petroleum jelly and cellophane tape.

Appropriate resolving gel mixture (12.5%) was prepared, and ammonium persulphate was added just before use. The clamped plate assembly was held vertically; and the resolving gel mixture was poured into the space between the glass plates, leaving sufficient space at the top for stacking gel. Care was taken to avoid any air bubbles in the gel. After polymerisation, the stacking gel (4%) mixture was prepared, poured over the resolving gel, and appropriate comb (with regard to the number of samples to be applied) was placed over it. The assembly was left undisturbed. After the polymerisation of stacking gel, the comb was carefully removed, and the wells were washed with the reservoir buffer, and fixed to the electrophoretic apparatus after removing the sealed cellophane tape and teflon spacer at the bottom.

Reservoir buffer was added to the lower and upper tanks of the electrophoretic apparatus. Care was taken to avoid air bubbles from the bottom of the gel to ensure uniform contact between the gel and the buffer.

**Sample Preparation and Sample Loading**

Four lens samples, each from experimental and control animals, were homogenised in 0.5 ml. of PBS solution (pH 7.4), centrifuged at 12,000 g, for
10 minutes. The supernatant was designated as the water-soluble-protein fraction. The residue was washed in PBS solution (pH 7.4) three times, and centrifuged at 12,000 g. for 10 minutes. The remaining residue was dissolved in 20 µl of 10% SDS, and was designated as water-insoluble-protein fractions.

The proteins were dissolved in sample buffer in the ratio 1:1 and incubated in a boiling water bath for two to three minutes, and chilled for one minute, cooled to room temperature. The sample was either used immediately; or stored in a refrigerator, and warmed just before use.

The protein sample was carefully loaded on to the gel surface using a microsyringe. Equal amount of protein was loaded in all the wells from different samples. Soon after loading the sample proteins, the power was switched on. The electrophoresis was done at a constant voltage of 50 V for 20 minutes followed by 100 V for five to six hours.

Standard SDS molecular weight markers (14, 18, 24, 34, 45 and 66 KDa) were used. After electrophoresis, the gel was removed from the apparatus; the glass plates and spacers were removed, and the gel developed by silver staining method.

**Silver Staining Method**

**Reagents and Chemicals**

A. 50 ml. methanol, 12.5 ml. glacial acetic acid, 0.5 ml. formaldehyde (Qualigens) were mixed and made upto 100 ml. with double-distilled water.

B. Sodium thiosulphate (BDH) solution: 20 g. was dissolved in 100 ml. double-distilled water.
C. 200 mg. silver nitrate (Qualigens) was dissolved in double-distilled water, added 0.07 ml. of formaldehyde, and made up to 100 ml. with double-distilled water, and kept in dark in a brown-coloured bottle.

D. Six g. of sodium carbonate was mixed with two ml. of solution B and 0.05 ml. formaldehyde, and made up to 100 ml. with double-distilled water.

E. 50% ethanol.

Procedure

Important - Used hand gloves always.

1. The gel was fixed in solution A for minimum one hour or overnight.
2. The gel was washed with 50% ethanol, three times, 20 minutes each.
3. The gel was treated with solution B for one minute.
4. The gel was washed in double-distilled water for three times of 20 seconds each.
5. The gel was incubated in solution C for 20 minutes in the dark.
6. The gel was washed in double-distilled water for three times of 20 seconds each.
7. The gel was developed with solution D until the bands were clear.
8. The gel was washed in double-distilled water twice for one minute each.
9. The gel was washed in 50% ethanol, and 10% acetic acid, for 10 minutes each.
10. Finally, the gel was washed in 50% ethanol, and stored in 15% ethanol. The gels were now ready for observation, and compared with reference to the standard protein markers used.
Chapter 3