MATERIALS AND METHODS
GSH-PO activity decreases rapidly with age in senile cataracts. This was more in nuclear cataract in comparison to cortical cataractous lenses (Fecondo and Augusteyn, 1983 and Rao et al., 1983). Bhat et al. (1991) observed a fall in the GSH-PO activity in brown lenses as compared to yellow lenses. A decrease in GSH-R activity was also observed in senile cataractous lenses (Rogers and Augusteyn, 1978; Rao et al., 1983; and Ohrloff et al., 1984; Rathbun and Bovis, 1986) and also in brown lenses (Bhat et al., 1991).

The concentration of GSH decreases rapidly in senile cataract prior to mature and in mature cataract (Harding, 1970; Reddy, 1971; Friedburg and Manthey, 1973; Truscott and Augusteyn, 1977a; and Brinkman and Broekhuyse, 1980). Low, Huang and Zigler (1989), Bando and Obazawa (1990), and Bhat et al. (1991) observed a decrease in GSH during the browning of the lens.

The literature till date has revealed that the senile cataract is evolved due to various risk factors and metabolic disturbance of the antioxidant defense system. Even though a number of studies have been carried out, none of them are able to explain the exact nature of the metabolic disturbance that leads to the formation of senile cataract. Therefore the information available at present is incomplete and deals either with the red cell
metabolism or the lens metabolism alone. It was also observed from the literature that it lacks an interrelationship between the two major metabolic systems such as "HMP shunt" and "glutathione linked system" of lens and erythrocytes, during cataractogenesis. Moreover, previous studies were concentrated on certain individual enzymes only and not a complete one. This was more so in case of enzymes transketolase and transaldolase. As regards to the other enzymes of "HMP shunt", enzymes of glutathione linked system were also incomplete in lens and erythrocytes during cataractogenesis.

Considering the above lacuna, the present experiment has been designed with a view to explore the possible roles of these enzymes of "HMP shunt" viz., glucose 6-phosphate dehydrogenase, transketolase, transaldolase along with the "antioxidant enzymes" like glutathione peroxidase, glutathione reductase, etc. in normal and cataractous cases of red blood cell as well as lenses in order to understand the biochemical mechanisms involved in senile cataract.
This study has been conducted to explore the possible biochemical mechanism involved in the formation of senile cataract with special reference to the enzymatic changes during the progress of senile cataract.

The enzymes of the HMP shunt and glutathione linked system of erythrocyte as well as lens have been studied. The other biochemical parameters studied in senile cataract were estimation of total proteins, soluble proteins and insoluble proteins of lens. The plasma glucose level was studied to check whether the subjects were diabetic or not.

The materials used were human blood and lens samples of normal controls of mixed population belong to the age group of 40-50 years and cataractous patients of mixed population with different stages of maturation viz., immature, mature, hypermature belong to the age group 40-85 years. The cataractous samples were obtained from the patients admitted for cataract extraction in the Ophthalmology Department, Medical College, Kottayam. Their clinical findings were recorded. Special attention has been paid to collect blood and lens samples from the same patients.
Normal control blood samples were collected from the hospital premises and nearby areas, who did not show any pathological signs of liver, heart or eye diseases. Human normal lens samples were obtained from the Eye Bank, Medical College, Kottayam. These eyes showed no opacification or cataract. Time interval between death and enucleation of eyes ranged from 1-6 h. The eyes were kept at 4°C and cornea used for keratoplast or preserved within 24 h after death. After the cornea was taken from donor eyes, the lenses were made available for biochemical analysis (Gupta and Mathur, 1983). The maximum time interval between death and biochemical analysis was 24 h. The postmortem changes in human lens due to time lapse between death and removal of lens have been assessed experimentally on rabbit eyes by Zinkham (1961) and reported that the activity of lens glucose 6-phosphate dehydrogenase was remarkably stable after death. This inference has been taken as valid for the enzymes in human beings too.

The subjects had undergone clinical examination under dilated pupil with ophthalmoscope and slit lamp and visual acuity in order to assess the stages of maturity i.e., immature, mature or hypermature cataract (Mathur, Goswammy and Agarwal, 1971; Agarwal, Gupta and Goswammy, 1976; Gupta and Mathur, 1983; and Chauhan, Desai, Bhatnagar and Garg, 1984).
Stages of cataract are defined as:

(a) **Immature cataract**

Visual acuity better than hand movement with counting fingers and presence of some optically clear space between the capsule and cataractous nucleus plus cortex.

(b) **Mature cataract**

Visual acuity, hand movement and accurate projection of light. All layers of the lens fiber opaque without any change in the capsule.

(c) **Hypermature cataract**

All hypermature cataract such as morgagnion cataract and all those showing deposition of calcium and cholesterol over the capsule, with visual acuity of accurate projection of light.

Collection and transportation of samples

(a) **Blood samples**

Venous blood specimens for the assays were anticoagulated with disodium-EDTA 20 mg per ml of blood. The samples were transported to laboratory in an ice bowl. Most of the samples were analysed promptly and the remaining samples were preserved at 4°C and assayed on the
next day. With use of EDTA as anticoagulant, enzymes were reported to be stable at 4°C for three weeks (Fairbanks and Klee, 1986).

(b) Lens samples

The lenses were extracted by intracapsular extraction so that the whole lens with intact capsule were obtained. The lenses thus obtained were collected in bottles with ice cold normal saline (0.9% sodium chloride solution). Immediately after the extraction, taken to the laboratory in an ice bowl and processed soon or later. In case of delay in processing, the lens samples were kept in frozen condition till the assay was carried out.

Preparation of freezing mixture

Mixed 1:1 crude salt and smashed ice cubes, kept this mixture in ice-box and used this for haemolysate preparation.

Preparation of haemolysate

Reagents

1. Sodium chloride (0.9%); kept at 4°C
2. Haemolyzing solution: Placed 10 ml of Na₂EDTA, 0.27 mol, pH 8.0 and 0.05 ml of 2-mercaptoethanol in a one litre volumetric flask and brought to one litre with distilled water. Stored at 4°C.
Procedure

Centrifuged 2 ml of whole blood at 1500 x g for 10 min. Removed plasma and stored for further analysis (Protein estimation). Buffy coat (the topmost cream coloured layer of cells) was removed completely. Erythrocytes were resuspended in 10 ml of cold sodium chloride and mixed thoroughly. Centrifuged at 1500 x g for 10 min. at 4°C. Removed and discarded supernatant and topmost layer of cells, including any residual buffy coat, without disturbing the uppermost layer of erythrocytes. Repeated the suspension of cells in cold sodium chloride, centrifugation, discarding of supernatant and uppermost layer of cells (to a depth of about 0.5 mm) three times. Resuspended the remaining cells in an equal volume of cold sodium chloride. Added 0.2 ml of the erythrocyte suspension to 1.8 ml of haemolyzing solution in a glass tube of 10 x 150 mm. Capped the tube and immersed it in freezing mixture until the contents were completely frozen (10 min.) and was thawed. Repeated the freeze thawing several times (preferably four times) until a crystal clear lysate was obtained. After the final freeze thawing it was mixed thoroughly. Transferred the tube into ice cold water and was maintained at 0°C till the assay. The haemolysate prepared in this way was referred as 1:20 haemolysate which was used throughout the experiment unless special type of haemolysate preparation is mentioned.
Preparation of lens homogenate

Lens samples were weighed on a Dhona analytical balance after drying within the folds of filter paper. Weighed samples were placed in a 15 ml Potter-Elvehjem glass grinder. 10 parts of chilled buffer or solutions were added (i.e., 2.2 ml for 0.22 g lens tissue) and homogenised with a teflon pestle. Enzyme assays were carried out on the supernatant obtained from the homogenate by spinning in a low temperature centrifuge at about 12000 x g for 10 min. The homogenate was prepared in this way and the supernatant obtained from this homogenate was used for all other experiments, unless mentioned.

Determination of glucose 6-phosphate dehydrogenase (G6PDH)
(Glucose 6-phosphate: NADP⁺ oxidoreductase EC 1.1.1.49)

G6PD activity of erythrocyte and lens samples were assayed by the method of Fairbanks and Klee (1986).

Reagents

1. a) Tris(hydroxy methyl)aminomethane-hydrochloride buffer hereafter called tris-HCl buffer (200 mM, pH 8.2 containing 50 mM EDTA-disodium salt).
b) Tris-HCl buffer (1.0 M, pH 8.0): 121.14 g of tris(hydroxy methyl)aminomethane (tris, trizma) was added to a one litre beaker, containing 800 ml double distilled water and mixed thoroughly. pH of the solution was adjusted to 8.0 by adding, 1N HCl, drop by drop with constant stirring. Transferred to a one litre volumetric flask and the volume was made upto one litre with double distilled water.

2. Magnesium chloride (0.1 M)
3. NADP⁺ (2 mM)
4. Stock reagent mixture: The above reagents were premixed sequentially, 0.5 ml each of buffer, magnesium chloride, NADP⁺ and 2.9 ml water for each experiment. The reagent mixture was prepared in advance as described above and stored in frozen condition (Stable upto one month).
5. Glucose 6-phosphate (6 mM), prepared on the day of assay in minimum volume or 0.3 ml for each sample and control.

Procedure

Two quartz cuvettes were marked as blank and sample. The sample cuvette was filled with 0.88 ml of stock reagent, 0.02 ml haemolysate or lens homogenate. The blank cuvette with 0.88 ml stock reagent and 0.1 ml double
distilled water. They were capped with parafilm, mixed well and incubated at 37°C for 10 min. Added 0.1 ml of glucose 6-phosphate solution to sample cuvette and capped with parafilm. Mixed well and placed in UV-visible spectrophotometer (Systronics 108) and the increase in absorbance measured at 340 nm. The measurement repeated after 10 min. Haemolysate-haemoglobin concentration was carried out by cyan-methemoglobin method (Varley, 1975). Protein concentration of lens homogenate was done by the modified method of Lowry (Dulley and Girieve, 1975).

Calculation

Enzyme activity ($E$) in international units per gram haemoglobin.

$$E = \frac{100 \times A}{Hb}$$

$A$ - Number of enzyme units per ml of sample

$Hb$ - Concentration of haemoglobin in grams per 100 ml of haemolysate

$$A = \frac{Ab \times Vc}{6.22 \times VH}$$

$Vc$ - Cuvette volume ($\mu$l)

$VH$ - Volume of sample ($\mu$l) the reaction system

$Ab$ - Absorbance changes per minute at 340 nm

6.22 - Optical absorbance of 1 mM of NAD(P)H
Calculation for haemolysate

G6PD activity IU/g Hb = \( \frac{100 A}{Hb} \)

\[ A = \frac{\Delta Ab \text{ 340/min.}}{6.22} \times \frac{1000}{20} \]

\[ A = 8.04 \times \Delta Ab \text{ 340/min.} \]

Therefore, \( 100 A = 8.04 \times 100 \Delta Ab \text{ 340/min.} \)

\[ = 804 \times \Delta Ab \text{ 340/min.} \]

Therefore, \( E = \frac{804 \times \Delta Ab \text{ 340/min.}}{Hb} \)

Calculation for lens

Enzyme activity \( E \) is expressed as IU/g protein

\[ E = \frac{100 A}{\text{gram protein/100 ml homogenate}} \]

\( A \) - Number of enzyme units/ml of homogenate

\[ A = \frac{\Delta Ab}{6.22} \times \frac{Vc}{VH} = \frac{\Delta Ab}{6.22} \times \frac{1000}{20} \]

\[ = \Delta Ab \times 8.04 \]

Therefore, \( 100 A = \Delta Ab \times 8.04 \times 100 \)
Therefore, \[ E = \frac{\Delta Ab \times 804}{\text{gram protein/100 ml homogenate}} \]

**Estimation of haemoglobin**

Estimation of haemoglobin was done by cyan-methemoglobin method of Varley (1975).

**Reagents**

1. Potassium ferricyanide
2. Potassium cyanide
3. Potassium dihydrogen phosphate
4. Noridet P40
5. Haemoglobin standard (50 mg%)

**Preparation of Drabkin’s solution**

Dissolved 50 mg potassium cyanide, 200 mg potassium ferricyanide and 140 mg potassium dihydrogen phosphate in 800 ml distilled water. 1 ml Noridet P40 was added to it, made to one litre with distilled water (pH was adjusted to 7.2), kept in a dark bottle at 4°C.

**Procedure**

Poured 9.8 ml Drabkin’s solution in to a test tube. 0.2 ml haemolysate was added to this and mixed thoroughly.
kept at room temperature for 4 min. Read the absorbance at 540 nm against Drabkin's solution. Read the absorbance of the standard solution as such against Drabkin's solution.

Calculation

Gram haemoglobin per 100 ml of haemolysate

\[
\frac{\text{Reading of test}}{\text{Reading standard}} = \frac{x \text{ Conc. of standard} \times \text{ dilution factor}}{x \text{ Conc. of standard}}
\]

Protein estimation

Total, soluble and insoluble proteins of lens were estimated by the modified method of Lowry (Dulley and Girieve, 1975). Soluble and insoluble fractions of the lens sample were separated as described by Satoh (1972) with slight modification developed in our laboratory (1% SDS was incorporated in the phosphate buffer used for washing).

Reagent

1. Potassium dihydrogen phosphate
2. Dipotassium hydrogen phosphate
3. Sodium lauryl sulphate (SDS)
4. Sodium hydroxide
5. Sodium potassium tartarate
6. Sodium carbonate
7. Copper sulphate
8. Folin-ciocalteu reagent

Preparation of reagents

1. Potassium phosphate buffer (50 mM, pH 7.2)
2. 1% SDS in potassium phosphate buffer
3. 0.1 N sodium hydroxide
4. 2% sodium potassium tartarate
5. 1% copper sulphate
6. 2% sodium carbonate solution in 0.1 N sodium hydroxide
7. Alkaline copper reagent (mixed 1 ml of 2% sodium potassium tartarate, 1 ml 1% copper sulphate and 100 ml 2% sodium carbonate solution containing 3% SDS).
8. Folin's reagent was diluted to 1 N with distilled water.

Preparation of soluble and insoluble protein fractions of lens

(a) Soluble content

Weighed lens after drying within the folds of filter paper on Dhona analytical balance. Prepared 10% homogenate
with proper amount of phosphate buffer (50 mM, pH 7.2) in a glass homogeniser in ice. Sufficient amount of 10% homogenate was taken for total protein estimation. Centrifuged the rest of the homogenate at 15,000 x g for 30 min. at low temperature and collected the supernatant. Redissolved the pellet in a total volume of 2 ml phosphate buffer containing 1% SDS, centrifuged and collected the supernatant. The process was repeated twice and the fractions were pooled. This constituted the soluble fraction of the lens protein.

(b) Insoluble content

Resuspended the pellet after the third centrifugation in a total volume of 2 ml 0.1 N NaOH and warmed at 60°C for half an hour with intermittent stirring. Collected the fully dissolved contents. Hereafter this was termed as insoluble fraction of lens proteins.

Procedure

Pipetted out 150 μl of the sample and 0.6 ml of alkaline copper reagent and incubated at 25°C for 10 min. After the incubation 60 μl of 1 N Folin's reagent was added and incubated at 25°C for 30 min. Measured the absorbance at 660 nm.
A calibration curve was prepared by using bovine serum albumin as standard. A range of 20 to 200 μg/ml was used for this. Linearity up to 120 μg/ml was obtained. Samples exceeding this limit in concentration were diluted accordingly.

Calculation

Protein concentration of aliquots of the sample was directly read from the calibration curve and calculated accordingly.

Protein concentration in plasma was expressed as g%. Protein concentration in lens sample was expressed as mg/g tissue.

Determination of transketolase activity (TK) (Sedoheptulose 7-phosphate: D-glyceraldehyde 3-phosphate glycolaldehyde transferase, EC 2.2.1.1)

TK activity of haemolysate and lens homogenate was assayed by the macro method of Myron Brin (1974).

Reagents for incubation

1. Sodium chloride
2. Potassium chloride
3. Dipotassium hydrogen phosphate
4. Hydrochloric acid
5. Magnesium sulphate
6. Ribose-5-phosphate (R-5-P monobarium salt)
7. Trichloroacetic acid
8. Sodium sulphate
9. Potassium hydroxide
10. D-glucose
11. Thiourea
12. Anthrone
13. D-ribose
14. Orcinol
15. Ferric chloride

Preparation of solutions

For incubation

1. Buffer (4 mM Na\(^+\), 115 mM K\(^+\): 20 mM PO\(_4\)^{3-}, Mg\(^{2+}\) 5 mM, pH 7.4): Mixed 40 ml 0.9% sodium chloride, 1030 ml 1.15% potassium chloride, 200 ml 1.75% dipotassium hydrogen phosphate solution (adjusted to pH 7.4 with 1 N HCl). 10 ml 3.82% magnesium sulphate was added to it. pH of the final solution was checked and adjusted to 7.4 with 1 N HCl.

2. Ribose 5-phosphate (R5 P-Barium salt) (47 mM): Dissolved 3.24 g R5 P-Ba in 8.5 ml 1N HCl, diluted to 45 ml with distilled water and slowly added 8 ml
saturated sodium sulphate solution. Allowed to precipitate and settle in the cold condition and centrifuged. Adjusted the pH of supernatant to 7.4 with 5 N KOH. Determined the ribose content (using orcinol reaction) and suitably diluted to 47 mM.

3. Trichloroacetic acid solution (7.5%): Dissolved 75 g TCA in distilled water and made upto 1000 ml.

For anthrone method

4. Hexose standard solution (0.55 mM): Dissolved 25 mg D-glucose in 25 ml distilled water, diluted 5 ml of this to 50 ml with 7.5% trichloroacetic acid solution. This solution contains 100 μg glucose/ml.

5. Anthrone reagent (2.5 mM anthrone and 0.13 molar thiourea): Dissolved 0.5 g anthrone and 10 g thiourea in 200 ml, 66% sulphuric acid by warming to 60-70°C. Allowed to cool and diluted to 1000 ml with 66% sulphuric acid. Kept for 24 h in the cold condition before use.

Orcinol method

6. Pentose standard solution (67 mM): Dissolved 25 mg D-ribose in 25 ml distilled water. Diluted 1 ml of this to 100 ml with 7.5% trichloroacetic acid solution, this solution contained 10 μg ribose/ml.
7. Orcinol reagent (14 mM orcinol and 0.062 mM ferric chloride): Dissolved 4.0 g orcinol and 335 mg ferric chloride in distilled water and made upto 100 ml, diluted to 2000 ml with 30% HCl.

**Determination of ribose content (with orcinol reaction)**

**Procedure**

Prepared three tubes and marked them as standard, test and blank. Pipetted out 1 ml pentose standard solution (67 mM) to the tube marked as standard, 1 ml ribose 5-phosphate stock, to the tube marked as test, 1 ml glass distilled water to the tube marked as blank. Pipetted out 4.5 ml orcinol reagent to all the three tubes, mixed thoroughly and placed the tubes for 20 min. in a boiling waterbath, cooled immediately to 0°C. Allowed to come to room temperature. Absorbance was measured at 620 nm and calculated the ribose content present in the solution.

**Preparation of samples**

(a) Blood samples

2 ml anticoagulated blood was centrifuged for 30 min. and removed the plasma. Equal volume of cold distilled water was added to the erythrocyte. Freezed immediately
by immersing the tubes containing erythrocyte in freezing mixture, thawed the samples by dipping the tubes in water at room temperature. Repeated the process three times. The lysate thus prepared was used for the estimation.

(b) Lens sample

Weighed the lens sample and homogenised in 10-fold volume of incubation buffer; transketolase activity was estimated in the homogenate.

Assay of enzyme by macro method (Anthrone method)

Pipetted out 0.5 ml sample into two tubes marked as blank and test, respectively. 0.65 ml buffer was added to the blank and 0.45 ml buffer to the test. Mixed well and 0.2 ml R 5-P solution was added to the test only. Mixed and incubated for 60 min. at 37°C. Reaction was terminated by adding 6 ml trichloroacetic acid solution to both the tubes. Mixed and centrifuged off the precipitate and used the supernatant for the assay.

Four test-tubes were marked as blank 1, blank 2, test and standard. Pipetted out 1 ml each of blank and test supernatants to blank 1 and test. Pipetted out 1 ml of 7.5% trichloroacetic acid solution into blank 2. Pipetted out 1 ml hexose standard solution into standard. 10 ml precooled anthrone reagent was added to all tubes with
constant stirring. Heated all the tubes for 10 min in a boiling waterbath, cooled for 5 min. in a cold waterbath. Allowed to stand for 20 min. in the dark and read extinction against blank 2 at 620 nm.

Calculations

Anthrone method

Volume of activity

\[
\text{Volume of activity} = \frac{\Delta E_{\text{Sample}} \times C_{\text{Standard}} \times 7.15 \times 1000}{\Delta E_{\text{Standard}} \times 60 \times 1.0 \times 0.5}
\]

\[
= 238 \times \frac{\Delta E_{\text{Sample}}}{\Delta E_{\text{Standard}}} \times C_{\text{Standard}} \text{ (U/l)}
\]

Where,

\(E\) is change in extinction

7.15 is the volume in ml after deproteinisation

60 is the incubation period in min.

1 is the volume of supernatant in ml used for assay

0.5 is the volume in ml of the original sample

\(C_{\text{Standard}}\) is the concentration in standard solution in \(\mu g/ml\) (0.55 mM)

Activity was expressed as U/l.
Determination of transaldolase activity (TA)
(Sedoheptulose 7-phosphate: D-glyceraldehyde 3-phosphate dihydroxyacetone transferase, EC 2.2.1.2)

TA activity was assayed by the method of Karl Brand (1983).

Reagents

1. Triethanolamine hydrochloride
2. Ethylenediaminetetra acetic acid-disodium salt
3. D-Fructose 6-phosphate (F6-P)
4. D-Erythrose 4-phosphate sodium salt (E4-P)
5. Reduced nicotinamide adenine dinucleotide (NADH)-disodium salt
6. Glycerophosphate dehydrogenase (GDH)
7. Triosephosphate isomerase (TIM)
8. Sodium hydrogen carbonate
9. Sodium hydroxide
10. Hydrochloric acid

Preparation of solution

1. Triethanolamine buffer (0.1 M, pH 7.6, 10 mM EDTA): Dissolved 1.857 g triethanolamine hydrochloride and 0.372 g EDTA-disodium salt in 80 ml distilled water, adjusted the pH to 7.6 with 1 N NaOH and diluted to 100 ml with distilled water.
2. Fructose 6-phosphate (0.15 M): Dissolved 105 mg fructose 6-P-Na salt (75% fructose 6-P) in 2 ml water.

3. Erythrose 4-phosphate (10 mM): Dissolved erythrose 4-phosphate in water and determined the erythrose 4-P concentration enzymatically as described by the method of Paoletti (1984) and diluted appropriately to 10 mM concentration with double distilled water. The pH of the solution was adjusted to 2.5.

4. Reduced nicotinamide-adenine dinucleotide (NADH: 7.5 mM): Dissolved 11 mg NADH, sodium salt, in 2 ml 1% sodium hydrogen carbonate solution.

5. Glycerophosphate dehydrogenase/triosephosphate isomerase (GDH: 30 KU/l, TIM: 200 KU/l): GDH (≥ 170 U/mg, 25°C) and TIM (≥ 10000 U/mg, 25°C) 1.8 mg GDH + 0.2 mg TIM suspended in 3.0 M ammonium sulphate solution. Diluted the mixture 10 times with 3.0 M ammonium sulphate solution.

Procedure

Pipetted out into the cuvette, 2.5 ml triethanolamine buffer, 0.5 ml F6-P solution, 0.05 ml erythrose 4-P solution, 0.05 ml NADH solution, 0.05 ml GDH/TIM suspension, sequentially. Mixed, waited for 2 min. and transferred 0.05 ml test sample into it. Read the absorbance at 339 nm. Repeated the reading after exactly 1, 2, 3, 4 and 5 min.
Calculation

\[ 8731 \times \frac{\Delta A}{\Delta t} \text{ (Change in absorbance/minute)} \]

Enzyme activity was expressed as U/L.

Assay of glutathione reductase (GSH-R)
(NADPH: oxidised glutathione oxidoreductase, EC 1.6.4.2)

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

Reagents and solutions

1. Phosphate buffer (0.12 M, pH 7.2 (37°C)): For one litre buffer, dissolved 16.33 g potassium dihydrogen phosphate in 800 ml water, adjusted the pH to 7.2 (at 37°C) with 1 N NaOH, 1 M and diluted to one litre with distilled water.

2. EDTA (15 mM): Dissolved 0.56 g EDTA-disodium salt in 100 ml water.

3. Flavin-adenine dinucleotide, FAD (155 μM): Dissolved 12.5 mg FAD, disodium salt, in 100 ml water.

4. Reduced nicotinamide-adenine dinucleotide phosphate (NADPH, 9.6 mM): Dissolved 8 mg NADPH, tetrasodium salt in 1 ml 1% sodium hydrogen carbonate solution.

5. Oxidised glutathione, GSSG (65.3 mM): Dissolved 40 mg GSSG in 1 ml distilled water.
Procedure

Pipetted out into the cuvette, 2.60 ml phosphate buffer, 0.01 ml EDTA, 0.10 ml FAD (in case of blood sample only), 0.10 ml GSSG and 0.10 ml test sample sequentially and mixed thoroughly, waited for 5 min and 0.05 ml NADPH was added. Mixed thoroughly and absorbance was taken for 10 min. with a time interval of 1 min.

Calculation

(a) Blood

Enzyme activity =

\[ 4.84 \times 10^3 \times \frac{\Delta A_t}{\Delta t} \text{ (Change in absorbance/min)} \]

where, \( 4.84 \times 10^3 \) is the factor used for calculating GSH-R activity by the assay method optimised for any source (phosphate buffer) at 339 nm, incorporating FAD in the system.

(b) Lens

Enzyme activity =

\[ 4.68 \times 10^3 \times \frac{\Delta A_t}{\Delta t} \text{ (Change in absorbance/min)} \]

where, \( 4.68 \times 10^3 \) is the factor used for calculating of GSH-R activity by the assay method optimised for any source (phosphate buffer) at wave length 339 nm.

Enzyme activity is expressed as U/l.
GSH-R activity of erythrocytes in gram haemoglobin

\[
\text{U/l} = \frac{\text{g Hb/l of sample}}{}
\]

GSH-R activity of lens in gram protein

\[
\text{U/l} = \frac{\text{gram protein/l of sample}}{}
\]

**Estimation of reduced glutathione level (GSH)**

Estimation of reduced glutathione level was measured by the method of Beutler, Duron and Kelly (1963).

**Reagents**

1. Glacial metaphosphoric acid
2. EDTA-disodium salt
3. Sodium chloride
4. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB)
5. Disodium hydrogen phosphate
6. Sodium citrate

**Preparation of reagents**

1. Phosphate buffer (0.12 M, pH 7.2)
2. Precipitating solution: Dissolved 1.67 g of glacial metaphosphoric acid, 0.2 g EDTA disodium salt and 30 g sodium chloride in 100 ml glass distilled water.
3. Sodium citrate (1%): Dissolved 1 g sodium citrate in 100 ml glass distilled water.

4. DTNB: Dissolved 20 mg of DTNB in 100 ml 1% sodium citrate solution.

5. Sham filtrate: It was prepared by diluting the precipitating solution in the ratio of 2:5.

6. 0.3 M disodium hydrogen phosphate

Preparation of samples

(a) Blood sample

0.2 ml blood was added to 2 ml distilled water, mixed rapidly. From the haemolysate thus formed 2 ml was taken for GSH estimation and 0.2 ml for haemoglobin estimation.

(b) Lens sample

Prepared 10% lens homogenate in phosphate buffer (0.12 M, pH 7.2 at 37°C) and used for estimation.

Procedure

2 ml sample was taken and 3 ml precipitating solution was added to it. Kept for 5 min. and filtered through a medium or coarse grade filter paper. From this filtrate 2 ml filtrate was added to 8 ml of 0.3 M disodium hydrogen phosphate solution in a 10 ml test tube. Absorbance (Ab₁)
was read at 412 nm against a blank prepared by adding 2 ml sham filtrate to 8 ml 0.3 M disodium hydrogen phosphate solution. 1 ml DTNB reagent was added to the blank and sample test tube, absorbance (Ab₂) read again at 412 nm.

Calculation

(a) Blood

Reduced glutathione (GSH) in micromoles per gram of haemoglobin (C) is obtained as follow.

\[
\frac{C}{1000} = \frac{(Ab₂ - Ab₁)E₁}{13600} \times \frac{11}{2a} \times \frac{5}{2b} \times \frac{100}{Hb}
\]

where, 13600 is the molar extinction coefficient of the yellow anion produced when GSH interact with DTNB.

11 - final volume of assay system
2a - amount of sample used
5 - volume of the diluted sample
2b - amount of filtrate
E₁ - correction factor for band width
Hb - concentration in g/100 ml lysate.

\[
C = \frac{Ab₂ - Ab₁ \times E₁ \times 101}{Hb}
\]
For lens the same formula was used by substituting gram protein per 100 ml homogenate instead of Hb.

Glutathione peroxidase (GSH-PO)  
(GSH-PO, EC 1.11.1.9)

GSH-PO activity was estimated by the method of Beutler (1975).

Reagents

1. Tris(hydroxy methyl)aminomethane-hydrochloride (Tris-HCl).
2. Ethylene diamine tetra acetic acid-disodium salt (EDTA)
3. Reduced glutathione (GSH)
4. Glutathione reductase 10 U/ml (GSH-R)
5. Reduced nicotinamide-adenine dinucleotide phosphate (NADPH)
6. t-Butyl hydroperoxide
7. Sodium hydroxide
8. 2-mercaptoethanol

Preparation of solutions

1. Tris-HCl buffer, 1 M (EDTA: 5 mM, pH 8.0): Dissolved 78.8 g tris-HCl and 0.931 gm EDTA in 250 ml glass
distilled water. Adjusted to pH 8.0 with 1 N NaOH. Made upto the volume to 500 ml with glass distilled water.

2. Glutathione reductase (10.5 U/ml): Dissolved the vial containing 105 units in 10 ml glass distilled water and kept the bottle in frozen state till use.

3. Reduced glutathione (GSH: 0.1 M): Dissolved 92 mg GSH in 3 ml distilled water.

4. Reduced nicotinamide-adenine dinucleotide phosphate (NADPH: 2 mM): Dissolved 10 mg NADPH in 6 ml glass distilled water.

5. t-Butyl hydroperoxidase (7 mM) approximately 1:1000 dilution.

6. 2-mercaptoethanol-EDTA stabilising solution: Prepared by bringing 0.05 ml of 2-mercaptoethanol and 10 ml of neutralised 10% (0.27 M) EDTA sodium salt solution to a volume of one litre with glass distilled water.

Procedure

Pipetted out into cuvette, 100 µl tris-HCl buffer, 20 µl GSH, 100 µl GSH-R, 100 µl NADPH, 10 µl sample supernatant and 670 µl glass distilled water sequentially. Incubated at 37°C for 10 min 10 µl t-butyl hydroperoxidase was added to the test sample and 10 µl glass distilled water to the blank. Absorbance was measured at 340 nm.
Additional blank

An additional blank was required for this system. Pipetted out 10 μl 2-mercaptoethanol-EDTA stabilising solution into assay system instead of test sample. Absorbance/minute was taken and subtracted this from the test sample absorbance/minute. This was done for nullifying the non-enzymatic oxidation of GSH and NADPH by t-butyl hydroperoxide.

Calculation

(a) Blood sample

GSH-PO activity in international unit (IU)/g Hb

\[ \text{IU/g Hb} = \frac{100 \Delta \text{Absorbance}}{\text{Hb}} \]

where, A is number of enzyme units per ml of sample and Hb is haemoglobin concentration in grams per 100 ml of haemolysate.

\[ A = \frac{\Delta \text{Absorbance}}{6.22} \times \frac{1000 \mu l}{10 \mu l} \]

where, \( \Delta \text{Absorbance} \) is change in absorbance, 1000 μl is the cuvette volume, 10 μl is the volume of haemolysate used in the assay system.

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(b) Lens sample

GSH-PO activity in international unit (IU)/g protein

\[
\text{100 A} = \frac{\text{gram protein/100 ml homogenate}}{} 
\]

Estimation of sugar

Estimation of blood sugar was done by the method of Cooper and McDaniel (1970).

Reagents

1. Thiourea
2. Glacial acetic acid
3. o-Toludine
4. D-Glucose
5. Benzoic acid

Preparation of reagents

1. o-Toludine reagent

Dissolved 1.5 g of thiourea in about 900 ml of glacial acetic acid in a one litre volumetric flask. 60 ml o-toludine was added to it and diluted to one litre with glacial acetic acid.
2. Benzoic acid solution 1.4 g/l (12 mM)

Dissolved 1.4 g of benzoic acid in 800 ml glass distilled water in a one litre volumetric flask with warming. Allowed to cool and then diluted to 1000 ml.

3. Glucose stock standard 1 g/100 ml (55.6 mM)

Dissolved 1 g of D-glucose in 80 ml benzoic acid solution in 100 ml volumetric flask and diluted with benzoic acid solution to 100 ml.

4. Glucose working standard 100 mg/100 ml (5.56 mM)

Diluted 5 ml of the stock standard to 50 ml in a volumetric flask with benzoic acid solution to obtain a concentration of 100 mg/100 ml.

Collection of sample

Blood sample was collected in the bulb containing sodium fluoride and potassium oxalate in the ratio of 1:3 (i.e., 20 mg of the mixture per 5 ml of blood). The sample was centrifuged and the plasma was used for sugar estimation.

Procedure

Three tubes were marked as blank, test sample and standard. Pipetted out 3 ml of o-toludine reagent to all
three test tubes. Pipetted out 50 μl test sample and 50 μl standard solution to their respective test tubes. Mixed the contents of each tube and heated in a boiling waterbath for 12 min. cooled, brought to room temperature and read at 630 nm.

A calibration curve was prepared using glucose standard of 50 to 300 mg%. Linearity was found upto the concentration of 300 mg%.

Calculation

Amount of glucose in the sample was calculated directly from the calibration curve prepared as above and expressed as mg/100 ml.

Statistical analysis

1. In order to compare different groups 't' test was used.

\[
t = \frac{\bar{X} - \bar{Y}}{\sqrt{\frac{n_1 s_1^2 + n_2 s_2^2}{n_1 + n_2 - 2} \left[ \frac{1}{n_1} + \frac{1}{n_2} \right]}}
\]

\(\bar{X}\) - Mean of first sample

\(\bar{Y}\) - Mean of the second sample

\(s_1^2\) - Variance of first sample
$s_2^2$ - Variance of second sample

$n_1$ - First sample size

$n_2$ - Second sample size

Follows t distribution with $n_1 + n_2 - 2$ df

2. To explore the relationship between two variables, Karl Pearson’s (1972) product movement correlation coefficient was worked out and it is tested for significance using t test.

$$t = \frac{s \sqrt{n-2}}{\sqrt{1-r^2}}$$

where,

$s$ - Sample correlation coefficient

$n$ - Number of pairs

Follows t distribution with $n-2$ df

3. To study the influence of age on cataract development, chi-square test of independence Brand and Snedecor formula (1972) was used.

where

$$\text{Chi-square} = \sum \frac{(O-E)^2}{E}$$

$$O = \frac{E \times R \times C}{N}$$

$$E = \frac{R \times C}{N}$$
Follows chi-square distribution with \((M-1) \times (n-1)\) df

where,

- \(O\) - Observed frequency
- \(M\) - Number of rows
- \(C\) - A column total
- \(R\) - A row total
- \(E\) - Expected frequency
- \(N\) - Grand total
- \(n\) - Number of columns
- \(df\) - Degree of freedom

**Chemicals and Solvents**

The chemicals used in the experiments were of research grade purity and were obtained locally or from BDH, Fluka, Sigma Chemicals.

1. Ammonium sulphate \((NH_4)_2SO_4\) (BDH)
2. Anthrone (Merck)
3. Benzoic acid (BDH)
4. \(t\)-Butyl hydroperoxidase (Sigma)
5. Copper Sulphate \((CuSO_4 \cdot 5H_2O)\) (Merck)
6. D-erythrose 4-phosphate sodium salt \((E4-P, Na Salt)\) (Sigma)
7. D-fructose 6-phosphate (F6-P) (Sigma)
8. D-glucose (Merck)
9. D-ribose (Sigma)
10. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) (BDH)
11. Ethylenediamine tetra acetic acid disodium salt (EDTA-Na₂H₂.2H₂O) (Polypharm)
12. Ferric chloride (FeCl₃.6H₂O) (Qualigens)
13. Flavin-adenine dinucleotide (FAD) disodium salt (Sigma)
14. Folin-ciocalteue reagent (SRL)
15. Glacial acetic acid (BDH)
16. Glacial metaphosphoric acid (CDH)
17. Glucose 6-phosphate (Sigma)
18. Glutathione reductase (GSH-R) (Sigma)
19. Glycerophosphate dehydrogenase (GDH) (Sigma)
20. Haemoglobin standard (Span)
21. Hydrochloric acid (HCl) (Merck)
22. Magnesium chloride (MgCl₂.6H₂O) (SRL)
23. Magnesium sulphate (MgSO₄.7H₂O) (Merck)
24. 2-Mercapto ethanol (Merck)
25. Nictoinamide adenine-dinucleotide phosphate (NADP⁺) (Sigma)
26. Noridet-P40 (Fluka)
27. Orcinol (SRL)
28. Oxidised glutathione (GSSG) (Sigma)
29. Potassium chloride (KCl) anhydrous (BDH)
30. Potassium cyanide (KCN) (Merck)
31. Potassium dihydrogen phosphate (KH2PO4) (Merck)
32. Potassium ferricyanide [K2Fe(CN)6] (BDH)
33. di-Potassium hydrogen phosphate (K2HPO4) (Merck)
34. Potassium hydroxide (KOH) (Merck)
35. Potassium sodium tartarate (COOK.CHOH.CHOH.COONa.4H2O) (Merck)
36. Reduced nicotinamide-adenine dinucleotide phosphate disodium salt (NADPH-Na2) (Sigma)
37. Ribose 5-phosphate monobarium salt (R5 P-monobarium salt) (Sigma)
38. Sulphuric acid (H2SO4) (Merck)
39. Sodium hydrogen carbonate (Na2CO3) anhydrous (Merck)
40. Sodium carbonate (Na2CO3) anhydrous (Merck)
41. tri-Sodium citrate (C6H5Na3O7.2H2O) (Merck)
42. Sodium chloride (NaCl) (Merck)
43. Sodium doedecyl sulphate (SDS) (SRL)
44. Sodium hydroxide (NaOH) (BDH)
45. di-Sodium hydrogen phosphate (Na2HPO4) (Merck)
46. Sodium hydrogen carbonate (NaHCO3) (Merck)
47. Sodium sulphate (Na2SO4.10H2O) (Merck)
48. Reduced glutathione (GSH) (Sigma)
49. Tris(hydroxymethyl)aminomethane (tris buffer) (SRL)
50. Tris(hydroxymethyl)aminomethane-hydrochloride (Tris hydrochloride) (SRL)
51. Thiourea (SRL)

52. O-Toludine (Merck)

53. Trichloro acetic acid (C₂HCl₃.O₂) (SRL)

54. Triethenolamine hydrochloride (SRL)

55. Triose phosphate isomerase (TIM) (Sigma)