*Biochem. J.* (1970) **117**, 957–960
*Printed in Great Britain*



# Free and Protein-Bound Glutathione in Normal and Cataractous Human Lenses

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(*Received* 27 *January* 1970)

Protein-bound glutathione was identified and measured in normal and cataractous human lenses. In a major group of cataractous lenses the bound glutathione concentration was higher than normal. Study of normal lenses showed that their glutathione content is age-dependent, decreasing steadily from about 3.5 $\mu$mol/g of lens at age 20 years to about 1.8 $\mu$mol/g of lens at age 65 years. Cataract brings further decreases.

The amount of GSH in the lens decreases in almost every type of cataract except the cataract caused by diquat in the rat (Pirie, Rees & Holmberg, 1969). Consequently the role of GSH in the maintenance of lens clarity has been of considerable interest. The possibility of GSH taking part in various interactions with proteins, including mixed-disulphide formation, has often been discussed (van Heyningen, 1962; Kinoshita, 1964). Herrmann & Moses (1945) identified GSH–protein mixed disulphides in rabbit and ox lenses more than 20 years ago, but further studies of these mixed disulphides were not carried out. In the present investigation, low-molecular-weight thiols bound to protein by disulphide links were identified and measured in normal and cataractous human lenses. The cataractous lenses were graded into four groups of increasing nuclear colour according to the system introduced by Pirie (1968). A preliminary account of this work has been published (Harding, 1969).

Protein–GSH and protein–cysteine mixed disulphides have been identified in human plasma albumin (King, 1961), in proteins of Ehrlich ascites cells (Révész & Modig, 1965; Modig, 1968), in bovine serum albumin (Modig, 1968) and in rat spleen, liver, heart, muscle, kidney and Yoshida ascites sarcoma (Jackson, Harrap & Smith, 1968). Streptococcal proteinase has a volatile thiol bound to the protein by a mixed-disulphide bond (Ferdinand, Stein & Moore, 1965). Papain has a disulphide-bound cysteine residue at its active site and hence requires activation by mercaptans, cysteine, cyanide, borohydride or other reducing agents (Klein & Kirsch, 1969; Sluyterman, 1967).

## METHODS AND MATERIALS

*Lenses.* Human cataractous lenses were those removed during cataract operations in the Oxford Eye Hospital. These lenses were classified into four groups as described by Pirie (1968). Only lenses from the two major groups were used (see Table 1). Normal human lenses were obtained at post-mortem; any with opacities were rejected.

*Isolation of protein-bound thiols (or the corresponding sulphonic acids).* Method 1. Each lens was decapsulated and homogenized in a Thunberg tube in air. A solution (2ml) of 0.1M-$NaHCO_3$ in 7M-urea, pH8.8, containing 1% (w/v) of iodoacetic acid (recrystallized) was placed in the stopper. The tube was evacuated on an oil pump. The stopper was closed to maintain vacuum and the iodoacetate was tipped on to the lens homogenate and the tube agitated vigorously to mix before being left at 20°C in the dark. After 3h the tube was opened and the contents were dialysed against 7M-urea, adjusted to pH4, for 18h. The contents of the dialysis sac were adjusted to pH 8.8 and reduced by the addition of 10mg of $NaBH_4$. Reduction proceeded for 1h at 20°C before 2ml of 10% (w/v) trichloroacetic acid was added to destroy excess of $NaBH_4$ and to deproteinize. After 20h at 20°C the suspension was centrifuged at 10000*g* for 20min and samples of the supernatant were taken for thiol determination and paper electrophoresis.

Method 2. Each lens was decapsulated, ground by hand in 10% (w/v) trichloroacetic acid in a Griffith's all-glass homogenizer. After 20h the precipitated protein was collected by centrifugation (10000*g* for 20min), washed three times with 10% trichloroacetic acid to remove free low-molecular-weight thiols and then dissolved in 2ml of 7M-urea at pH9. Sodium borohydride (10mg) was added and the solution was kept at 20°C for 1h before addition of 2ml of 10% trichloroacetic acid (and a drop of pentan-1-ol) to destroy excess of borohydride and to deproteinize. After 20h the suspension was centrifuged (10000*g* for 20min) and samples of the resultant supernatant were taken for thiol determination and paper electrophoresis.

Method 3. The lens proteins, obtained by trichloroacetic acid precipitation as described above, were oxidized with freshly prepared performic acid (Moore, 1963) for 20h at 4°C. The solution was deproteinized with trichloroacetic acid and the supernatant used for paper electrophoresis.

*Thiol determination.* Thiols were measured by using the 5,5′-dithiobis-(2-nitrobenzoic acid) reagent of Ellman (1959) as described by Sedlak & Lindsay (1968), except

that the scale was halved and 1M-tris buffer, pH9.0, replaced the 0.4M-tris buffer, so that determinations could be carried out on trichloroacetic acid supernatants without prior neutralization. Complete recovery of GSH added to trichloroacetic acid supernatants was obtained by using this method.

*Paper electrophoresis.* This was carried out at 10V/cm, usually for 3h on a ridge-pole apparatus on Whatman no. 52 paper in pyridine–acetate buffer, pH4 (Grassmann, Hannig & Plöckl, 1955), or a buffer of formic acid–acetic acid–water, pH1.6 [125ml of 90% (v/v) formic acid and 375ml of acetic acid in 2.5 litres].

The method of removal of trichloroacetic acid from the solutions before electrophoresis is described below.

In some cases the sulphonic acids produced in method 3 were measured after electrophoresis and development in ninhydrin–cadmium acetate (Heilman, Barollier & Watzke, 1957) by using a strip-reader spectrophotometer (550nm; Beckman DU). The sulphonic acid of GSH was generously supplied by Dr S. G. Waley.

*Diagonal paper electrophoresis.* Trichloroacetic acid extracts of lenses were freed from an excess of trichloroacetic acid by extraction with *N*-methyldioctylamine (British Hydrological Corporation, Merton, Surrey, U.K.) in chloroform (5%, v/v), and the aqueous layer was extracted with chloroform to remove excess of base (Calam & Waley, 1963). After rotary evaporation the solution was subjected to diagonal paper electrophoresis, pH1.6 for each direction, with oxidation by performic acid on paper between electrophoresis runs (Brown & Hartley, 1966).

## RESULTS

The results of determinations of free and protein-bound thiols are presented in Table 1. Most determinations of protein-bound thiol followed isolation of the thiol by method 1; but measurements with method 2 and the 5,5′-dithiobis-(2-nitrobenzoic acid) reaction, or method 3 followed by paper electrophoresis, development with ninhydrin and spectrophotometric determination gave similar results. Each determination of protein-bound thiol required two normal or group I lenses, but a single group II lens was sufficient.

Paper electrophoresis (both one-dimensional and diagonal) showed that almost all the free low-molecular-weight thiol in the normal human lens is GSH. This applies equally to most of the cataractous lenses, but in a few cataractous lenses there was evidence of more cysteine than usual. Determinations of free low-molecular-weight thiol therefore may be considered as determinations of GSH with little error.

Study of normal human lenses showed that their free GSH content is age-dependent (Fig. 1), decreasing steadily from about 3.5μmol/g of lens at age 20 years to about 1.8μmol/g at age 65 years (this is the average age of the cataract lenses used). Cataract brings a further decrease to about 1.3μmol/g in a group I cataract and 0.9μmol/g in a group II cataract. The GSH content of cataractous lenses is not dependent on age. The differences of free thiol content between the group I and group II lenses and the mean value for normal lenses of age over 45 years are statistically significant.

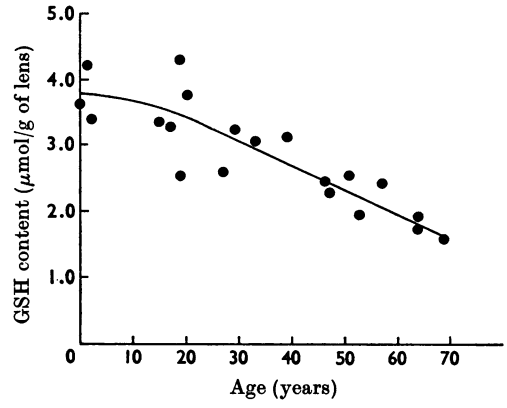


Fig. 1. Free GSH content of normal human lens.

The major low-molecular-weight thiol bound to protein in normal human lenses was shown to be GSH by paper electrophoresis at pH1.6 and 4.0 after isolation of the thiol or its sulphonic acid as described in the Methods and Materials section. In some cataractous lenses protein-bound cysteine (up to 40% of the total protein-bound thiol in group II lenses) was also found.

There is no more protein-bound low-molecular-weight thiol in cataractous lenses of group I (cortical cataracts without a sharply defined nucleus) than in normal human lenses (Table 1). The difference in bound-thiol content between normal and group II cataract lenses is statistically significant.

The loss of free low-molecular-weight thiol from the cataractous lens (at the stage of a group II cataract) is approx. 0.9μmol/g (subtracting the group II value from the normal value at age 65 years), compared with the aging loss of 1.6μmol/g between the ages of 20 and 65 years in normal lens. The free GSH lost with aging or with formation of a group I cataract is not bound to protein, but more than half of the loss of free GSH in the group II cataractous lens can be found as a mixed disulphide with protein.

## DISCUSSION

Published values for the GSH content of normal human lenses vary from 1.0 to 11.4μmol/g of lens (Mach, 1966; Barber, 1968; Dickinson, Durham & Hamilton, 1968; Dische, 1968; Consul & Nagpal,

Table 1. *Free and protein-bound thiol of human lens*

Numbers of determinations are given in parentheses. Two lenses/determination were used for protein-bound thiol determinations in normal and group I lenses.

| | Appearance | % of cataractous lenses in group | Thiol content (μmol/g wet wt. of lens) | |
|---|---|---|---|---|
| | | | Protein-bound thiol | Free thiol |
| Normal group | Clear | — | 0.46 (7) | —* |
| Cataract group I | Uniform yellow | 45 | 0.45 (5) | 1.3 (7) |
| Cataract group II | Pale cortex, visible nucleus | 42 | 0.82 (7) | 0.9 (9) |

* About 1.8 in lens from 60–70-year-old (see Fig. 1).

1968). The present results would indicate a value of about 2.9 μmol/g at age 37 years compared with 2.2 μmol/g found by Dickinson *et al.* (1968). The range of GSH contents of normal human lens reported now is similar to that found previously for monkey lens, but lower than that in the lenses of other animals (Reddy, 1967).

In the present work it has been shown for the first time that the GSH content of normal human lens is age-dependent, decreasing from about 3.5 μmol/g of lens at age 20 years to about 1.8 μmol/g at age 65 years (Fig. 1). In rabbits, rats and cattle there is an initial sharp increase in GSH concentration in the very young lens, but after maturity it remains almost constant (Bellows, 1944; Pirie, van Heyningen & Boag, 1953; Pirie, van Heyningen & Flanders, 1955; Sippel, 1962; A. Pirie, unpublished work). Between these two stages there is a slight decrease of GSH concentration in rats (Bellows, 1944; Sippel, 1962) and a tenfold decrease in rabbits (Pirie *et al.* 1953, 1955; A. Pirie, unpublished work). There was no evidence of the initial sharp increase of GSH content in young human lenses in the present work (Fig. 1). The free GSH content is approx. 10% of the protein thiol content of a normal human lens (J. J. Harding, unpublished work).

The GSH content of lenses decreases in most types of cataract. In human cataract a lowered GSH content has been found by Mach (1966), Dische (1968) and by Consul & Nagpal (1968), whereas it has been reported that cataractous lenses have GSH contents varying both above and below normal (Barber, 1968; Dickinson *et al.* 1968).

The present results clearly demonstrate a decreased concentration of GSH in two major groups of cataractous lenses (including 87% of all cataractous lenses removed in Oxford in 1966). The only previous author to classify lenses on the basis of colour found almost as much variation in the GSH content (above and below normal values) in each group as in the entire pool of cataractous lenses (Barber, 1968). In the present work only one out of nine group II lenses had a GSH content higher than the lowest normal value found (Fig. 1). The differences between each group of cataract lenses and normal lenses was statistically significant.

The presence of protein-bound GSH in normal human lens and an increased binding in at least 42% of all cataractous lenses (group II) adds a further factor to discussion of lens GSH and its role in the maintenance of lens clarity. During aging there is a net loss of free GSH from the normal human lens while the protein-bound GSH remains constant. During cataract formation there is a further net loss of GSH, as well as an increased binding of GSH to lens protein (Table 1).

The protein-bound GSH (PSSG) is probably in an equilibrium expressed by the equation:

$$PSSG + GS^- \rightleftharpoons PS^- + GSSG$$

(The methods of determination described immediately stop such interchange.) Thus under normal conditions free GSH keeps the protein-bound GSH at a low concentration, the GSSG formed being reduced back to GSH by NADPH and glutathione reductase. In cataract some part of the system governing GSH–GSSG interchange and formation and breakdown of GSH or both has changed resulting in a low concentration of GSH in the lens. The primary alteration is unknown but could relate to glutathione reductase or its coenzyme, NADPH. Under these changed conditions increased amounts of GSH become bound to protein. Huisman & Dozy (1962), working on haemoglobin, predicted that GSH would become bound to protein in conditions where its concentration was abnormally low. In human cataract at the stage of a group II lens about half the GSH present is bound to protein by disulphide bonds.

There are very few, if any, protein–protein disulphide links in normal human lens but about 4 μmol of 'half-PSSP'/g in group II cataractous lenses (J. J. Harding, unpublished work). It appears therefore that in cataract certain protein

thiol groups become oxidized, some forming cross-links and others binding GSH.

The author is grateful to Dr A. Pirie for helpful suggestions and discussion throughout this work, to his colleagues in the Nuffield Laboratory of Ophthalmology for advice and encouragement and to Mr K. C. Rixon for technical assistance. The support of the Medical Research Council is gratefully acknowledged.

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