Altered Heat-Lability of a Fraction of Glutathione Reductase in Aging Human Lens

By JOHN J. HARDING

*Nuffield Laboratory of Ophthalmology, University of Oxford, Walton Street,
Oxford OX2 6AW, U.K.*

(Received 19 March 1973)

An unusually heat-labile fraction of glutathione reductase appears in human lens at about an age of 32 years. No such change was found in glucose 6-phosphate dehydrogenase nor in 3-phosphoglycerate kinase. The change is an intrinsic property of glutathione reductase. A greater proportion of the altered glutathione reductase was found in the core, the older part, of the lens. No evidence of a second band of activity was obtained after electrophoresis. Possible interpretations of the results, including errors of protein synthesis, production of a new isoenzyme and post-synthetic changes, are discussed.

Holliday & Tarrant (1972) showed that unusually heat-labile fractions of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase accumulate in human fibroblasts in culture during the last quarter of their life span. The authors concluded that their results supported Orgel's theory of aging, which suggests that in aging errors occur in protein synthesis leading to production of proteins with abnormal sequences. Enzymes involved in protein synthesis would be affected in the same way, causing additional errors and finally an error catastrophe and death (Orgel, 1963).

The bulk proteins of some cataractous human lenses differ in conformation from those of normal human lens (Harding, 1972). The experiments of Holliday & Tarrant (1972) indicated a way of investigating whether altered, but still active, enzymes occur in the lens with the possibility of learning more about aging of the lens, cataract and the relationship between the two. Lewis & Tarrant (1972) showed that enzyme proteins that were inactive but had retained immunological identity also accumulate in aging cell cultures.

In the present work three enzymes in human lenses with and without cataract were examined for changes in thermal stability with age.

Materials

Commercial materials

Yeast glutathione reductase (EC 1.6.4.2), glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12), NADPH, NADP⁺, NADH, ATP, glucose 6-phosphate and 3-phospho-D-glycerate were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K.; GSSG from Sigma (London) Chemical Co.,

Kingston upon Thames, Surrey, U.K.; phenazine methosulphate from Calbiochem Ltd., London W.1, U.K. BDH Chemicals Ltd., Poole, Dorset, U.K., supplied 2,6-dichlorophenol-indophenol, Thiazolyl Blue (MTT) and most common chemicals.

Human lenses

Lenses referred to as 'normal' were clear lenses obtained *post mortem*. Human cataractous lenses were those removed in the Oxford Eye Hospital. They were graded as described by van Heyningen (1972).

Methods

Extraction of enzymes

Glutathione reductase. Lenses were ground in five times their weight of 67 mM-phosphate buffer, pH 6.6, containing 43 mM-K⁺ and 48 mM-Na⁺ (Horn, 1963); insoluble material was removed by centrifugation at 10000g for 20 min at 20°C. The supernatant was diluted tenfold with the same buffer.

Glucose 6-phosphate dehydrogenase. Intact pairs of lenses were ground in five times their weight of water; insoluble material was removed by centrifugation at 10000g for 20 min at 20°C. To 1.8 ml of the supernatant was added 3.6 ml of water and 0.6 ml of a solution containing 10 mM-EDTA, 10 mM-mercapto-ethanol and 0.2 M-NADP⁺.

Phosphoglycerate kinase. Intact lenses were ground in five times their weight of 0.1 M-triethanolamine hydrochloride, pH 7.6; insoluble material was removed by centrifugation at 10000g for 20 min at 20°C. Then 0.2 ml of the supernatant was diluted with a further 1.8 ml of 0.1 M-triethanolamine hydrochloride, pH 7.6.

Assays of the enzymes

Glutathione reductase (EC 1.6.4.2) activity was measured by the procedure of Horn (1963), as modified by Harding (1973).

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) activity was measured by the procedure of Löhr & Waller (1963).

Phosphoglycerate kinase (EC 2.7.2.3) activity was assayed by following the back reaction coupled with glyceraldehyde phosphate dehydrogenase (Bücher, 1955) as described in the information leaflet of Boehringer Mannheim (G.m.b.H.), Mannheim, Germany.

Glutathione reductase

Studies of thermal stability. The extract (6ml) described above was incubated at a fixed temperature ($70.0 \pm 0.2^\circ\text{C}$ or $66.5 \pm 0.2^\circ\text{C}$) for 30 min. Samples (0.6ml) were removed before incubation (t_0) and at subsequent intervals. The samples were cooled and centrifuged on a bench centrifuge to remove precipitated protein and 0.4ml of each supernatant was taken for assay of glutathione reductase.

In some experiments a mixture (1:1, v/v) of extracts from two lenses of different ages was used to study the effect of components of each extract on the thermal stability of glutathione reductase from subjects of different ages.

Another set of experiments was carried out on the central portion (nucleus) and outer portion (cortex) of lenses separately.

Effect of NADP⁺, NADPH, GSSG, GSH and cysteine on the thermal stability of glutathione reductase. Thermal-stability experiments were carried out as described above for glutathione reductase at 70°C but in the presence of each of the following: 0.1 mM-NADP⁺, 0.1 mM-NADPH, 1 mM-GSSG, 10 mM-GSH or 10 mM-cysteine.

Thermal stability of yeast glutathione reductase alone. Yeast glutathione reductase (0.1 ml of suspension having 1 mg/ml and 90 units/mg) was added to 9.9 ml of 67 mM-phosphate buffer, containing 43 mM-K⁺ and 48 mM-Na⁺, pH 6.6 (Horn, 1963). This solution was incubated at 56°C for 30 min. Two 10 μl samples were removed at zero time and further 10 μl samples were removed at intervals thereafter. Glutathione reductase activity was measured in these samples.

Thermal stability of yeast glutathione reductase in the presence of lens extracts. Lens extracts were prepared as described above. Yeast glutathione reductase (0.1 ml of a suspension containing 9 units) was added to the total extract (about 10 ml) of each lens. The solutions were incubated at 56°C and 10 μl samples removed at zero time (two samples) and subsequent intervals. Glutathione reductase activity was measured in the samples.

Electrophoresis

Starch-gel electrophoresis. The methods of Kaplan (1968) were used for the electrophoresis of lens extracts and for staining part of the gel. As the stain was not specific for glutathione reductase, corresponding parts of unstained gel were cut out, frozen and then extracted with 67 mM-phosphate buffer, containing 43 mM-K⁺ and 48 mM-Na⁺, pH 6.6 (Horn, 1963). These extracts were assayed for glutathione reductase activity over 45 min.

Polyacrylamide-gel electrophoresis. The methods and apparatus (Shandon Southern Instruments Ltd., Camberley, Surrey, U.K.) described by Hauschild-Rogat & Smith (1968) were used. The gel buffer and electrode vessel buffers both contained 70 mM-Tris, 7 mM-EDTA (disodium salt) and 10 mM-boric acid at pH 8.7 (Ferris *et al.*, 1964) and at pH 9.0. The eluting buffer contained 67 mM-phosphate, 43 mM-K⁺ and 48 mM-Na⁺, pH 6.6, and had a flow rate of 14 or 19 ml/h; water extracts of six human lenses were loaded on to the gel. Fractions (3.5 or 4.7 ml) of effluent were assayed for glutathione reductase. A preliminary electrophoretic run of 1 h was required to avoid spurious results in the assay of the early fractions.

Thermal-stability study of glucose 6-phosphate dehydrogenase

Lens extracts of glucose 6-phosphate dehydrogenase were incubated at 50°C for 30 min. At zero time and subsequent intervals 0.6 ml samples were removed for assay of glucose 6-phosphate dehydrogenase.

Thermal-stability study of phosphoglycerate kinase

The diluted extracts (see above) were incubated at 48.8°C for 30 min. Two 50 μl samples were removed at zero time and further 50 μl samples were removed at intervals after that for assay of phosphoglycerate kinase.

Results

Glutathione reductase

The inactivation at 70°C of lens glutathione reductase from subjects near the extremes of the human life span is shown in Fig. 1. The extract from a 91-year-old lost 18% of its activity whereas that from a newborn subject lost only 5.6% of its activity. No more inactivation occurred after 15 min; consequently it appears that the percentage inactivation is also the percentage of unusually heat-labile enzyme. Percentage inactivation in 30 min was plotted against age (Fig. 2). Up to at least 20 years of age there was only 3–6% inactivation and no age-related trend. From the age of 38 to 91 years there was about 24% inactivation with no trend related to either age or cataract. Between 20 and 38, apparently occurring at an age of 31 to 32 years,

Fig. 1. Inactivation at 70°C of glutathione reductase obtained from lens of a newborn (●) and 91-year-old (■) human

For details see the text.

Fig. 2. Percentage inactivation of lens glutathione reductase in 30 min at 70°C as a function of age

●, Normal lenses (clear, post-mortem); ■, cataractous (group I); ▲, cataractous (group II).

there was a sudden increase in the unusually heat-labile fraction. The difference between the means of inactivation of glutathione reductase from young lenses (age <25) compared with older lenses (age >35) was highly significant (Student's *t* test, *P* < 0.001) (see Table 1). Both sexes were represented in each age group.

The results of inactivation experiments carried out at 66.5°C are plotted in Fig. 3. At 66.5°C there was no inactivation of glutathione reductase in extracts of lenses from subjects aged 32 years or less, but 15–22% (mean 18.6%) inactivation in extracts from subjects aged 41–93 years, including one cataractous lens.

The results at 66.5°C confirm the view that there is an abrupt loss of heat-stability of part of glutathione reductase related to age. The combined results suggest that this transformation occurs suddenly at about the age of 32 years. The activity of glutathione

Table 1. Inactivation of human lens glutathione reductase kept at 70°C for 30 min

Results are expressed as means ± s.d. for the numbers of determinations given in parentheses. The differences between the means are significant (Student's *t* test, *P* < 0.01 for mixtures compared with older lenses, *P* < 0.001 for the other two comparisons).

	% inactivation in 30 min at 70°C
Age less than 25 years	4.6 ± 1.0 (5)
Age greater than 35 years	24.0 ± 4.6 (11)
Mixture of young and older lenses	15.1 ± 4.2 (5)

Fig. 3. Percentage inactivation of lens glutathione reductase in 30 min at 66.5°C as a function of age

●, Normal lenses; ▲, cataractous (group II).

reductase in human lens changes little with age in the adult (J. J. Harding, unpublished work).

The experiments with mixtures of supernatants from young (under 25 years) and older (over 35 years) lenses gave overall inactivation in 30 min at 70°C of 15.1 ± 4.2% (see Table 1); this differed significantly from the mean for the younger lenses (Student's *t* test, *P* < 0.001) and from the mean for the older lenses (Student's *t* test, *P* < 0.01). Further, the mean inactivation for the mixtures approximated to the mean of the results for the younger group and older group (14.3%).

The results on thermal inactivation of lens glutathione reductase from separate nuclear and cortical regions at 70°C are shown in Table 2. Cortical material from two lenses was used in each experiment; nuclear material from two or four lenses was used for each experiment. All material was from lenses of subjects aged over 35 years. In this age-range mean inactivation in whole lens is 24% under the same conditions (Table 1). Glutathione reductase from the cortical region suffered only about 14% inactivation

Table 2. *Inactivation of human lens glutathione reductase in extracts from nuclear and cortical regions kept at 70°C for 30 min*

For details see the text.

Portion of lens	Age (years)	Number of lenses used	Wt. taken of each lens (mg/lens)	% inactivation in 30 min	Normal or cataractous
Cortex	55	2	91	12	Normal
	57	2	102	15	Normal
	80 and 89	2	65	14	Cataractous (Group II)
Nucleus	55 and 57	4	114	33	Normal
	45	2	95	39	Normal
	86	2	65	40	Normal
	80 and 89	2	162	30	Cataractous (Group II)

Table 3. *Inactivation of lens glutathione reductase (from people aged over 35 years) kept at 70°C for 30 min in the presence of NADPH, NADP⁺, GSH, GSSG or cysteine*

For details see the text.

Compound	Concn. (mM)	% inactivation after		
		2 min	5 min	30 min
Control	0	5	14	24
NADPH	0.1	80	—	—
NADPH	0.1	77	92	—
NADP ⁺	0.1	—	14	17
GSH	10	—	30	66
GSH	10	—	26	66
GSSG	1	—	10	15
GSSG	1	—	19	24
Cysteine	10	—	18	43

whereas that from the nuclear region suffered 30–40% inactivation (Table 2). Thus there was a greater proportion of the unusually heat-labile enzyme in the nuclear region. Even so, the cortical region of older lenses has a greater proportion of the more heat-labile enzyme than the whole young lens.

Yeast glutathione reductase was more heat-labile than lens enzyme and its inactivation had to be studied at 56°C in the presence or absence of lens extracts. Alone it suffered 45% loss of activity in 30 min; in the presence of extract from young (13-year) lens it lost 40% of its activity; in the presence of extract from older (66-year) lens it lost 33% of its activity.

The results of inactivation of lens glutathione reductase at 70°C in the presence of NADPH, NADP⁺, GSH, GSSG and cysteine are given in Table 3. GSSG and NADP⁺ have no effect on the thermal stability; cysteine and GSH appear to decrease thermal stability somewhat, but the major effect found was the considerable de-stabilization by 0.1 mM-NADPH. De-stabilization of erythrocyte glutathione

Table 4. *Distribution of glutathione reductase activity from young (23-year) and older (65-year) human lenses in starch gels after electrophoresis*

Fractions are numbered in order of increasing mobility.

Fraction	% of recovered activity	
	23-year-old	65-year-old
1	0	4
2	4	18
3	66	50
4	30	28
5	0	0

reductase by NADPH has been observed by Icén (1967).

Electrophoresis of glutathione reductase

The results of starch-gel electrophoresis and assays to locate glutathione reductase are shown in Table 4. The enzyme from both young (23-year) and older (65-year) lenses was found mainly in the same single fraction, although the activity from the older lens may be more diffuse. There is no evidence of a second separated band of activity in the older lens.

Polyacrylamide-gel electrophoresis also showed only a single component of the enzyme in extracts of lenses from subjects aged 50–70 years.

Glucose 6-phosphate dehydrogenase

The inactivation at 50°C of lens glucose 6-phosphate dehydrogenase in the presence of NADP⁺, EDTA and mercaptoethanol is shown in Fig. 4. No difference was found in the thermal stability of the enzyme from lenses of subjects aged 2–89 years, with or without cataract. Glucose 6-phosphate dehydrogenase in extracts of lens from newborn subjects

Fig. 4. Inactivation at 50°C of glucose 6-phosphate dehydrogenase from lens

Inactivation plots from seven experiments on extracts from lenses of humans aged from 2 to 89 years, including two cataractous lenses (groups II and III) fell within the hatched area. The points (●) mark the mean of three inactivation plots for lenses of newborn humans.

appeared to be a little more stable than that from older lenses.

3-Phosphoglycerate kinase

The stability of 3-phosphoglycerate kinase at 48.8°C showed no decrease with age between 0 and 87 years. Two extracts of cataractous lenses (Group II) gave results similar to the normal lenses.

Discussion

The results described (Figs. 2 and 3; Table 1) show that there is a sudden increase, at about the age of 32 years, in an unusually heat-labile fraction of glutathione reductase from human lens. No such change was found in glucose 6-phosphate dehydrogenase (Fig. 4) nor in 3-phosphoglycerate kinase. Holliday & Tarrant (1972) found similar increases, near the end of culture life, in the unusually heat-labile fraction of two enzymes from human fibroblast cultures. They concluded that the unusually heat-labile enzymes are a result of erroneous incorporation of amino acids into proteins during synthesis.

Is the change in thermal stability of lens glutathione reductase an intrinsic property of the enzyme or is it

caused by environmental changes? The possibility of an abrupt environmental change in the human lens at the age of about 32 years seems unlikely. Cysteine, GSH and particularly NADPH de-stabilize the enzyme (Table 3) but the content of GSH in the lens is falling slowly at this stage (Harding, 1970). Little is known of the concentrations of cysteine and NADPH in human lens except that both are low. The experiments with yeast glutathione reductase showed that its inactivation was not promoted by extracts from older lenses.

The results on thermal stability of lens glutathione reductase in mixed extracts (Table 1) show more conclusively that the change at about 32 years is a property of the enzyme. The enzyme of older lenses did not seem to be stabilized by the extract of young lenses, nor did the enzyme of young lenses seem to be de-stabilized by the older extracts.

An intrinsic change in the enzyme could occur in several ways. It is conceivable that a change in the proportions of isoenzymes of glutathione reductase occurs at about 32 years. This seems unlikely for several reasons. Isoenzymes of glutathione reductase have been sought but not found (Kaplan, 1968; Brewer, 1970). Changes of isoenzyme patterns mostly occur during early development and are usually associated with rapid environmental changes, e.g. birth. The electrophoretic results (Table 4) indicate that the lens probably does not suddenly start the synthesis of a new unusually heat-labile isoenzyme at 32 years of age.

The electrophoretic results do not support the view that an abrupt specific post-synthetic change such as deamidation has occurred in glutathione reductase. Nevertheless this negative evidence is not conclusive as some single deamidations are not readily revealed by conventional electrophoresis (Midelfort & Mehler, 1972). Post-synthetic deamidation of glutamine and asparagine occurs in some proteins (Bloemendal *et al.*, 1972; Delcour & Papaconstantinou, 1972; Flatmark & Sletten, 1968; Midelfort & Mehler, 1972). These deamidations are continuous changes. The abrupt transformation of glutathione reductase in the lens is unlikely to be the result of a similar process.

The possibility that the altered glutathione reductase in lenses of humans aged over 35 years is a result of somatic mutation seems unlikely for the reasons discussed by Holliday & Tarrant (1972).

The possibility remains that the sudden alteration in glutathione reductase in the human lens at an age of about 32 years is the result of errors during protein synthesis as Holliday & Tarrant (1972) suggested for the changes they observed. Certainly only this interpretation accounts for the suddenness of the change.

On the basis of Orgel's theory (Orgel, 1963) the reported results would indicate that by the age of

35 years about 20% of the active glutathione reductase has non-specific errors of sequence causing thermal instability. The proportion of mis-translated enzyme would almost certainly be higher because whereas some errors would produce active, heat-stable enzyme, others, especially multiple errors, would produce inactive enzyme. The levelling off of the proportion of unusually heat-labile enzyme from age 35 years could mean that mis-translation has reached a plateau. In the original paper Orgel (1963) suggested that once mis-translation started to increase it would continue to increase exponentially, but later (Orgel, 1970) he concluded that in some circumstances the error frequency could rise rapidly and then level off. Relative rates of synthesis and breakdown of 'normal' and unusually heat-labile glutathione reductase could also lead to a steady proportion of heat-labile enzyme.

Finding a greater proportion of altered glutathione reductase in the nucleus (Table 2) gives some support to the view that this fraction is a product of post-synthetic changes, because less protein synthesis occurs in human lens nucleus than in cortex (Agarwal *et al.*, 1972; see also Wannemacher & Spector, 1968). Even a low rate of protein synthesis would be enough to give rapid turnover of a number of enzymes, including glutathione reductase, if there was no turnover of bulk protein. If, however, the alteration in glutathione reductase is caused by post-synthetic changes, the electrophoretic results indicate that the changes are not specific.

A post-synthetic change would be expected to be continuous. An alteration in some aspect of protein synthesis (translational errors, isoenzyme changes etc.) would be expected to produce altered enzyme in the cortex rather than the nucleus although it could conceivably do the reverse.

I am grateful to Dr. A. Pirie and Dr. R. van Heyningen for helpful suggestions and discussions, to my other colleagues at the Nuffield Laboratory of Ophthalmology for

advice and encouragement and to Mr. K. C. Rixon for skilled technical assistance.

References

- Agarwal, L. P., Gupta, S. & Goswamy, S. (1972) *Orient. Arch. Ophthalmol.* **10**, 174–187
- Bloemendal, H., Berns, A. J. M., van der Ouderaa, F. & de Jong, W. W. W. (1972) *Exp. Eye Res.* **14**, 80–81
- Brewer, G. J. (1970) *An Introduction to Isoenzyme Techniques*, pp. 113–115, Academic Press, London and New York
- Bücher, T. (1955) *Methods Enzymol.* **1**, 415–422
- Delcour, J. & Papaconstantinou, J. (1972) *J. Biol. Chem.* **247**, 3289–3295
- Ferris, T. G., Easterling, R. E. & Budd, R. E. (1964) *Anal. Biochem.* **8**, 477–486
- Flatmark, T. & Sletten, K. (1968) *J. Biol. Chem.* **243**, 1623–1629
- Harding, J. J. (1970) *Biochem. J.* **117**, 957–960
- Harding, J. J. (1972) *Biochem. J.* **129**, 97–100
- Harding, J. J. (1973) *J. Chromatogr.* **77**, 191–199
- Hauschild-Rogat, P. & Smith, I. (1968) in *Chromatographic and Electrophoretic Techniques* (Smith, I., ed.), vol. 2, pp. 475–484, Heinemann, London
- Holliday, R. & Tarrant, G. M. (1972) *Nature (London)* **238**, 26–30
- Horn, H. D. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 875–879, Academic Press, London and New York
- Icén, A. (1967) *Scand. J. Clin. Lab. Invest.* **20**, Suppl. **96**, 1–67
- Kaplan, J. C. (1968) *Nature (London)* **217**, 256–258
- Lewis, C. M. & Tarrant, G. M. (1972) *Nature (London)* **239**, 316–318
- Löhr, G. W. & Waller, H. D. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H.-U., ed.), pp. 744–751, Academic Press, London and New York
- Midelfort, C. F. & Mehler, A. H. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 1816–1819
- Orgel, L. E. (1963) *Proc. Nat. Acad. Sci. U.S.* **49**, 517–521
- Orgel, L. E. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 1476
- van Heyningen, R. (1972) *Exp. Eye Res.* **13**, 136–147
- Wannemacher, C. F. & Spector, A. (1968) *Exp. Eye Res.* **7**, 623–625