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Glutathione Peroxidase in Lens and a Source of Hydrogen Peroxide in Aqueous Humour

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1. Glutathione peroxidase has been demonstrated in cattle, rabbit and guinea-pig lenses. 2. The enzyme will oxidize GSH either with hydrogen peroxide added at the start of the reaction or with hydrogen peroxide generated enzymically with glucose oxidase. 3. No product other than GSSG was detected. 4. Oxidation of GSH can be coupled with oxidation of malate through the intermediate reaction of glutathione reductase and $NADPH_2$. 5. Traces of hydrogen peroxide are present in aqueous humour: it is formed when the ascorbic acid of aqueous humour is oxidized. 6. Hydrogen peroxide will diffuse into the explanted intact lens and oxidize the contained GSH. The addition of glucose to the medium together with hydrogen peroxide maintains the concentration of lens GSH. 7. Glutathione peroxidase in lens extracts will couple with the oxidation of ascorbic acid. 8. It is suggested that, as there is only weak catalase activity in lens, glutathione peroxidase may act as one link between the oxygen of the aqueous humour and $NADPH_2$.

Glutathione peroxidase (glutathione–hydrogen peroxide oxidoreductase; EC 1.11.1.a), which catalyses the oxidation of glutathione with hydrogen peroxide:

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

was first described by Mills (1957) in erythrocytes. Mills (1960) and Cohen & Hochstein (1963) have suggested that, in erythrocytes, this reaction is more important for the removal of hydrogen peroxide than the breakdown of hydrogen peroxide by catalase. Since the catalase activity of lens is weak (Zeller, 1953) it is conceivable that glutathione peroxidase may play an important part in lens metabolism not only by removing hydrogen peroxide but also by giving rise to GSSG. Kinoshita & Masurat (1957) have shown that, under certain conditions, the addition of GSSG to lens extracts stimulates oxidation of glucose by the pentose phosphate path. The reactions involved (Scheme 1) are: (*a*) reduction of GSSG by glutathione reductase ($NADPH_2$–glutathione oxidoreductase; EC 1.6.4.2) and $NADPH_2$; (*b*) reduction of the NADP so formed by reaction with glucose 6-phosphate and glucose 6-phosphate dehydrogenase. Mills & Randall (1958) and Cohen & Hochstein (1963) showed that glucose 6-phosphate dehydrogenase will couple with GSH peroxidase of liver and erythrocytes.

van Heyningen & Pirie (1953) described glutathione reductase in lens, and Kinoshita (1955, 1964) has shown that the pentose phosphate path of glucose oxidation is operative in lens.

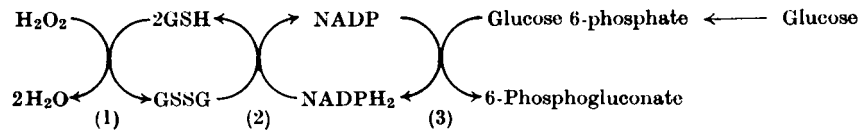
Glutathione peroxidase has been found in the lenses of cattle, rabbits and guinea pigs. Enzyme activity can be demonstrated not only with added hydrogen peroxide but also with hydrogen peroxide generated enzymically by glucose oxidase. Possible sources of hydrogen peroxide are examined and discussed.

METHODS

Lenses. Cattle and calf lenses were removed within 0·5–2·0 hr. of death and used at once or after being kept at −20°. Guinea-pig lenses were removed immediately on death from an overdose of intraperitoneally injected Nembutal. Rabbit lenses were removed and used immediately after death or after storage at −20°.

Lens extracts. These were prepared by grinding the lenses in a mortar with a little washed sand until a smooth dispersion was obtained. Distilled water or 0·9% NaCl was added to give a 1:4 (w/v) dilution and the suspension was centrifuged for 20 min. at 10000*g*. When necessary, dialysis was carried out in cellophan tubes against large volumes of distilled water for 24–48 hr. in the cold room. The tubes were shaken every 12 hr. and the water was changed. In this way nearly all the GSH was removed from the lens extract.

Materials. GSH was a gift from The Distillers Co. Ltd., Speke, Liverpool. NADP was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. H_2O_2 (30 vol. %) and 2,6-dichlorophenol-indophenol were obtained from British Drug Houses Ltd., Poole, Dorset. Glucose oxidase, peroxidase, catalase and glutathione reductase were



Scheme 1. (1) GSH peroxidase; (2) GSSG reductase; (3) glucose 6-phosphate dehydrogenase.

from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Determination of GSH. GSH was estimated spectrophotometrically, with a Unicam SP.500 spectrophotometer, by the method of Grunert and Phillips (1951).

Determination of GSSG. This was carried out spectrophotometrically with $NADPH_2$ and glutathione reductase (Bergmeyer, 1963). The reaction went to completion and the amount of GSSG present was calculated from the change in extinction at 340mμ. $NADPH_2$ was prepared by the method of Straub (1942).

Determination of ascorbic acid. This was carried out in two ways. (*a*) In aqueous humour and lens extracts. Proteins were precipitated with metaphosphoric acid (final concn. 2–3%), and ascorbic acid in the protein-free supernatants was titrated with 2,6-dichlorophenol-indophenol made up so that 1·0ml. of dye$\equiv$0·1mg. of ascorbic acid. The end point of the titration is a faint pink. (*b*) In aqueous humour. A spectrophotometric method was used when the presence of H_2O_2 was subsequently to be investigated. A known volume (0·1–0·3ml.) of standard 2,6-dichlorophenol-indophenol was pipetted into a 1cm. spectrophotometer cell, and 1·0ml. of 0·1M-phosphate buffer, pH6·6, and water to 2·8ml. were added. After thorough mixing, the extinction of the dye was measured at 600mμ. A sample of aqueous humour (0·05–0·3ml.) was then added, well mixed, and a further reading at 600mμ was made. The difference between the two readings is directly proportional to the ascorbic acid present. A standard graph was constructed by using a standard solution of ascorbic acid.

Detection of hydrogen peroxide in aqueous humour. A test described by Mapson (1945) was used. This depends on the reoxidation to a blue dye of leuco-2,6-dichlorophenol-indophenol by H_2O_2 in the presence of peroxidase. Ascorbic acid was estimated in the sample as described above (*b*). The mixture in the spectrophotometer cell was then stirred with a polythene rod and a final reading taken. A trace of undiluted peroxidase was added on the end of the rod and a further reading made. An increase in extinction at 600mμ after addition of peroxidase was taken to indicate the presence of H_2O_2. Controls showed that addition of catalase caused no change in reading, and the subsequent addition of peroxidase caused only a slight rise. This test was done at pH6·6 since peroxidase is active at this pH and the preliminary reduction of the dye by ascorbic acid is rapid. A similar test for the presence of H_2O_2 in aqueous humour can be done qualitatively on a larger scale by making a visual comparison of the intensity of the blue colour of the dye before and after addition of peroxidase. If the dye is just completely reduced by the added ascorbic acid then the colour change will be from white to blue if H_2O_2 is present.

Activity of glutathione peroxidase. This was determined by estimating the rate of loss of GSH in the presence of lens extract and H_2O_2 compared with its rate of loss in the presence of H_2O_2 alone (Cohen & Hochstein, 1963). EDTA (final concn. 25μg./ml.) and, in some cases, sodium azide (final concn. 0·4mg./ml.) were added to lens extract and to a fresh neutralized solution of GSH of approximately the same concentration (2mM) as that in the lens extract. Samples of lens extract and of GSH solution were pipetted into centrifuge tubes. At zero time, metaphosphoric acid (final concn. 2–3%) was added to one pair of samples and H_2O_2 (final concn. 1mM) to the other samples, which were held at room temperature. At different times, the reaction was stopped by addition of metaphosphoric acid. All tubes were left at 4° for at least 1hr., then centrifuged to remove protein and GSH was estimated in the supernatant solutions. In some experiments H_2O_2 was added to the total volume of lens extract and GSH solution and samples were removed by pipette at intervals thereafter into metaphosphoric acid.

Oxygen uptake. This was measured in conventional Warburg manometers at 20° in air.

Buffers. Krebs–Ringer phosphate and Krebs phosphate buffers were prepared according to Umbreit, Burris & Stauffer (1949).

RESULTS

Experiments were set up to determine the rate of oxidation of GSH by hydrogen peroxide in neutral solution with and without the addition of an extract of lens. The concentration of GSH in the lenses of most mammals is 5–10mM, which makes it unnecessary to add GSH to undialysed extracts. Table 1 shows that lens extract accelerates the removal of GSH by hydrogen peroxide; GSH in lens extract alone is stable during short periods. A solution of GSH, freshly prepared and neutralized, and of the same concentration as in the lens extract, was used to determine the rate of the non-enzymic oxidation of GSH by hydrogen peroxide. EDTA was added to all tubes to inhibit the metal-catalysed oxidation of GSH (Cohen & Hochstein, 1963), in spite of the fact that Pirie & van Heyningen (1954) found that EDTA increased the oxidation of GSH in undialysed lens extracts, during 2–5 days at 4°, owing to its catalysis of the oxidation of the ascorbic acid present, which was then non-enzymically reduced by GSH. The concentration of EDTA used in the present experiments was between 1% and 5% of that used in the earlier ones, and, as the duration of the present determinations of glutathione peroxidase seldom exceeded 30min., the oxidizing effect of EDTA was considered negligible.

In early experiments sodium azide was added to

Table 1. *Activity of glutathione peroxidase in lens*

EDTA (final concn. 25 μg./ml.) and sodium azide (final concn. 0·4 mg./ml.) were added to a freshly prepared extract of lens and to a fresh, neutralized solution of GSH of approx. the same concentration (2 mM) as that of the lens extract. Samples (0·25 ml.) were pipetted into centrifuge tubes, which were kept at room temperature. Then 1·5 ml. of 2·5% metaphosphoric acid was added to zero-time samples and water or H_2O_2 was added to further tubes as follows: (*a*) Lens control: 0·25 ml. of lens extract, EDTA and azide + 0·1 ml. of water; (*b*) Lens + H_2O_2: 0·25 ml. of lens extract, EDTA and azide + 0·1 ml. of 2 mM-H_2O_2; (*c*) GSH and H_2O_2: 0·25 ml. of GSH, EDTA and azide + 0·1 ml. of 2 mM-H_2O_2. The reaction was stopped at intervals after the addition of substrate by the addition of 1·5 ml. of 2·5% metaphosphoric acid. The tubes were chilled for at least 1 hr., centrifuged to remove the proteins and GSH was estimated in the supernatant by the method of Grunert & Phillips (1951).

Species and final dilution of lens	Time (min.)	Loss of GSH (μmole/sample)		
		Lens control	Lens + H_2O_2	GSH + H_2O_2
Calf (1:7)	2	0	0·085	0
	5	0	0·18	—
	25	0	0·25	0·07
Cattle lens-cortex (1:8)	1	—	0·15	0
	5	—	0·38	0
	20	—	0·50	0·04
Guinea pig (1:8)	0·5	—	0·04	0·01
	5	—	0·25	—
	30	—	0·29	0·06

Table 2. *Effect of dialysis and of the omission of sodium azide on the activity of glutathione peroxidase of lens*

Expt. 1: EDTA (final concn. 25 μg./ml.) and sodium azide (final concn. 0·4 mg./ml.) were added to a 1:4 extract of cattle lens-cortex held for 48 hr. at 2° undialysed, or dialysed against several changes of distilled water at 2° for the same time. GSH was added to the dialysed extract to give a final concn. of approx. 2·5 mM. Zero-time samples (0·5 ml.) were precipitated with 1·5 ml. of 2·5% metaphosphoric acid, then 0·4 ml. of 8·5 mM-H_2O_2 was added to 5·5 ml. each of dialysed and of fresh lens extract, EDTA and azide. Samples were taken into metaphosphoric acid at intervals and GSH was estimated in the supernatants by the method of Grunert & Phillips (1951). Expt. 2: A non-dialysed extract of lens (1:4) was used as described above with and without added azide.

Expt. no.	Time (min.)	Loss of GSH (μmoles/ml. of incubation mixture)	
		Dialysed for 48 hr.	Undialysed
1	0·5	0·09	0·25
	1·5	0·42	0·48
	3	0·59	0·68
	5	0·69	0·89
		With azide	No azide
2	1	1·29	1·45
	3	1·72	1·65

inhibit catalase, but it was later omitted as it was found to make no difference to the rate of loss of GSH (Table 2). Dialysis of lens extracts for 24–28 hr. at 2° did not markedly affect their activity; in fact it was an advantage, since the GSH content of all reaction mixtures could then be standardized (Table 2). Fig. 1 shows that loss of GSH is roughly proportional to the amount of lens added. These results may be considered to demonstrate the presence of glutathione peroxidase in lens.

In these first experiments hydrogen peroxide was added at the start and the concentration fell during the experiment through the activity of glutathione peroxidase and possibly through other reactions. To see whether glutathione peroxidase of lens was capable of removing hydrogen peroxide generated in an enzymic reaction, glucose oxidase and glucose were used to form hydrogen peroxide and the changes in GSH were then studied in the presence and absence of lens extract. The reaction was followed manometrically and, in separate experiments, GSH was estimated at intervals. Keilin & Hartree (1955) showed that the addition of catalase to glucose and glucose oxidase halved the oxygen uptake, catalase

releasing 1 atom of oxygen from each molecule of hydrogen peroxide formed. Glutathione peroxidase decomposes hydrogen peroxide with the formation of water and no release of oxygen, so that the uptake in the presence of lens should be the same as in its absence, and double that in the presence of catalase. Fig. 2 records such an experiment. In this all GSH had been lost in the presence of lens and 90% from the flask containing the glucose-oxidase system and GSH alone. In another experiment of 15min. duration, lens, GSH and the glucose-oxidase system took up 6·1 μmoles of oxygen and used 10·5 μmoles of GSH, whereas the system without lens took up 6·0 μmoles of oxygen and used 4·1 μmoles of GSH. Oxygen uptake by the glucose-oxidase system alone was 5·7 μmoles and in the presence of catalase 3·9 μmoles. Table 3 records further experiments showing that, with enzymically generated hydrogen peroxide, the rate of disappearance of GSH depends on the concentration of lens. Glutathione peroxidase will therefore react with hydrogen peroxide produced either enzymically or non-enzymically.

Products of the glutathione-peroxidase reaction. It has been accepted that glutathione peroxidase forms GSSG, but a balance sheet of the reaction has not so far been published. Experiments with lens extract together with glucose oxidase and glucose as source of hydrogen peroxide showed rough equivalence between oxygen uptake, GSH loss and GSSG formation. With the use of crude lens extracts, other

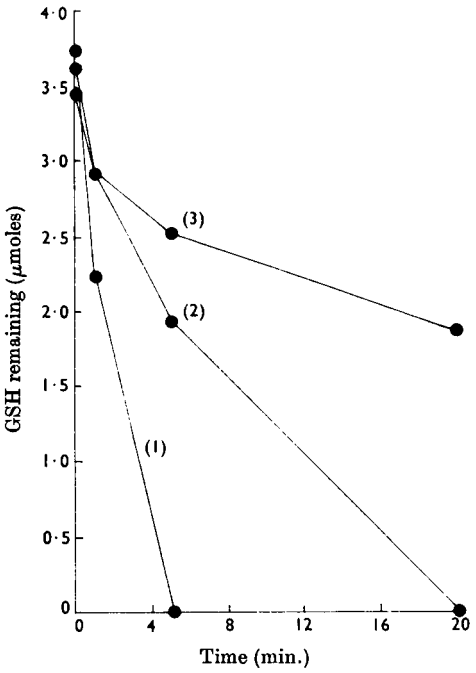


Fig. 1. Loss of GSH with added H_2O_2 and different amounts of lens. Cattle lens-cortex (1:5 extract) was dialysed for 24hr. at 2° and then to 5·0ml. was added 0·5ml. of sodium azide (4mg./ml.) and 0·5ml. of EDTA (0·25mg./ml.), to give mixture A. Incubation mixtures were set up as follows: (1) 1·0ml. of mixture A, 0·3ml. of Krebs phosphate buffer, pH 7·4, and 0·15ml. of GSH (10mg./ml.); (2) 0·5ml. of mixture A, 0·3ml. of Krebs phosphate buffer, pH 7·4, 0·15ml. of GSH (10mg./ml.) and 0·5ml. of water; (3) 0·25ml. of mixture A, 0·3ml. of Krebs phosphate buffer, pH 7·4, 0·15ml. of GSH (10mg./ml.) and 0·75ml. of water. Zero-time samples (0·3ml.) of each were added to 1·5ml. of 2·5% metaphosphoric acid; 0·4ml. of 5mM-H_2O_2 was added to the remaining solutions and 0·3ml. samples were taken at intervals.

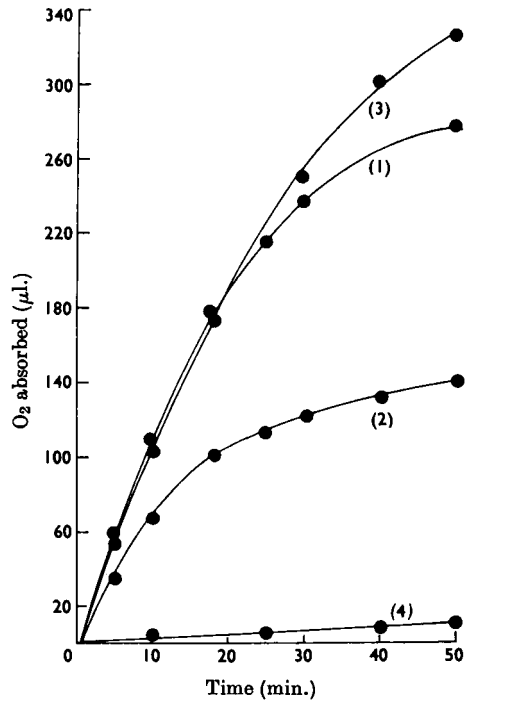


Fig. 2. Oxygen uptake of glucose-oxidase system, with catalase and with lens and GSH.

Manometer no.	(1)	(2)	(3)	(4)
Glucose (30mg./ml.) in side arm	0·1	0·1	0·1	—
Glucose oxidase (10mg./ml.)	5 μl.	5 μl.	5 μl.	—
Lens (1:4 extract)	—	—	1·0	1·0
GSH (10mg./ml.)	—	—	0·3	0·3
EDTA (0·25mg./ml.)	0·3	0·3	0·3	0·3
Krebs phosphate buffer, pH 7·4	2·6	2·6	1·3	1·4
Catalase (10mg./ml.)	—	5 μl.	—	—

The final volume in all flasks was 3ml. The temperature was 20°, the gas phase was air and KOH was placed in the centre wells of the manometer cups.

Table 3. *Reaction of glutathione peroxidase of lens with hydrogen peroxide generated by glucose oxidase*

GSH control: the incubation medium consisted of 3·0 ml. of 0·2 M-potassium phosphate buffer, pH 6·9, 3·0 mg. of glucose, 2·5 mg. of GSH, 0·1 ml. of 0·1 M-EDTA and Krebs–Ringer phosphate to 5·0 ml. Lens: different volumes of cattle or rabbit lens extract replaced an equal volume of Krebs–Ringer phosphate in the GSH control mixture. All tubes were slowly shaken at 20°. Zero-time samples (0·2 ml.) were deproteinized by the addition of 0·8 ml. of 2·5% metaphosphoric acid. Then 20 μg. of glucose oxidase (5 μl.) was added to each tube. Samples (0·2–0·5 ml.) were taken at intervals and deproteinized, and GSH was estimated in the supernatants by the method of Grunert & Phillips (1951).

Time (min.)	Loss of GSH (μmoles/ml. of incubation mixture)		
	GSH control	Cattle lens (1:13)	Cattle lens (1:39)
4	0·03	0·46	0·13
10	0·06	0·63	0·30
20	0·23	1·1	0·63
10	0·15	1·1	—
20	0·31	1·6	—
	GSH control	Rabbit lens* (1:13)	Rabbit lens* (1:39)
5	0·0	0·68	0·18
10	0·046	0·96	0·52
15	0·14	1·52	0·80

* Prepared from lenses kept frozen for 2 years.

Table 4. *Balance sheet of oxidation of glutathione by glutathione peroxidase of lens and glucose oxidase*

The balance sheet refers to the following reactions:

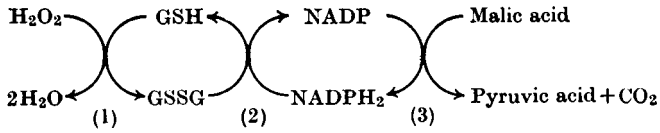
$$\text{Glucose} + O_2 \rightarrow H_2O_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2H_2O$$

$$1 \text{ mole} \equiv 1 \text{ mole} \equiv 2 \text{ moles} \equiv 1 \text{ mole}$$

Uptake of O_2 was measured manometrically. At the end of the experiment 1·0 ml. of fluid was removed and added to 2·0 ml. of 2·5% metaphosphoric acid to precipitate proteins. Zero-time samples were similarly precipitated. GSH was estimated in the supernatant, after centrifugation, by the method of Grunert & Phillips (1951). GSSG was estimated in the supernatants by determining the oxidation of $NADPH_2$ at 340 mμ on the addition of glutathione reductase (Bergmeyer, 1963). For the GSH control incubation conditions were: 3 or 10 mg. of glucose was placed in the side arms of the manometer flasks; 1·3 ml. of 0·2 M-phosphate buffer, pH 6·9, 0·3 ml. of EDTA (0·25 mg./ml.), 5 μl. (20 μg.) of glucose oxidase, 0·3 ml. of GSH (10 mg./ml.) and water to 3·0 ml. were placed in the main flask; KOH and paper were in the centre wells. With the lens the incubation conditions were as above, but 1·0 ml. of 1:4 extract of cattle lens-cortex was added in Expts. 1 and 2 and 1·0 ml. of 1:3 chopped lens-cortex in Expt. 3 in place of 1·0 ml. of water.

Expt. no.	Time (min.)	Total change with lens (μmoles)		
		O_2 uptake	GSSG increase	GSH decrease
1	15	6·1	4·2	10
2	25	3·0	3·6	11·2
3	20	1·5	1·9	4·0
		Total change without lens (μmoles)		
1	15	6·0	2·0	4·0
2	25	3·0	1·8	4·0
3	20	1·2	0·6	1·6

reactions, apart from the ones investigated, will almost certainly occur. Table 4 shows, first, that more GSH is lost and more GSSG is formed in the presence of lens than in its absence, which confirms the activity of glutathione peroxidase in lens. The oxygen absorbed is approximately equivalent to the GSSG formed but the loss of GSH in Expt. 2 is greater than expected. Search for other products of oxidation was made by electrophoresis on paper of the protein-free supernatants of the reaction mix-



Scheme 2. (1) GSH peroxidase; (2) GSSG reductase; (3) malic enzyme.

tures at pH 4·0 with a pyridine–acetic acid buffer (15 ml. of pyridine and 50 ml. of acetic acid in 2·5 l. of water), but no ninhydrin-positive spots other than those of GSH and GSSG were found, apart from the normally occurring amino acids of lens.

Coupling of glutathione peroxidase with malic enzyme of lens. The glutathione-peroxidase system can function effectively only if there is a means of regenerating GSH. I therefore investigated the coupling of glutathione peroxidase, through the intermediate reaction of glutathione reductase, with an enzyme forming $NADPH_2$. Mills & Randall (1958) and Mills (1960) showed that glutathione peroxidase of erythrocytes and of liver will couple with glucose 6-phosphate dehydrogenase. van Heyningen & Pirie (1953) found an active malic enzyme [L-malate–NADP oxidoreductase (decarboxylating); EC 1.1.1.40] in lens and showed that it could be coupled with glutathione reductase, which is also present in lens (Scheme 2).

One would expect therefore that the addition of malate and NADP would decrease the loss of GSH in the presence of hydrogen peroxide and extract of lens. Table 5 shows that, when hydrogen peroxide is added to lens, malate and NADP there is an immediate loss of GSH followed by a slow increase. Such increase does not take place in the absence of malate and NADP. This shows that coupling of glutathione peroxidase with other enzymes of lens that form $NADPH_2$ is possible.

Sources of hydrogen peroxide. A source of hydrogen peroxide is necessary for glutathione-peroxidase activity. One obvious source is the ascorbic acid of aqueous humour and lens. In the eyes of most mammals the concentration of ascorbic acid in aqueous humour, vitreous humour and lens is far higher than in blood, being about 1·0 mM. It is mainly in the reduced form but a small amount of dehydro-L-ascorbic acid is present in both aqueous humour and lens. There are only traces of GSH in aqueous humour (Pirie & van Heyningen, 1956). At the pH of the aqueous humour, which is 7·5–7·6 in the rabbit (Kinsey, 1953), ascorbic acid is autoxidizable to dehydro-L-ascorbic acid and hydrogen peroxide (Steinman & Dawson, 1942; Mapson, 1945). In fact Mills & Randall (1958) used ascorbic acid as oxidant in their study of glutathione peroxidase in erythrocytes. Oxidation of ascorbic acid is catalysed by copper, traces of which have been found in cattle aqueous humour by Bowness, Morton, Shakir & Stubbs (1952). Oxidation is also catalysed by riboflavine in the light (Hopkins, 1938). Aqueous humour was therefore examined to see whether hydrogen peroxide could be detected and whether it was formed when the ascorbic acid of aqueous humour was oxidized.

Table 5. *Effect of malate and nicotinamide–adenine dinucleotide phosphate on the rate of loss of glutathione in the presence of hydrogen peroxide and lens extract*

With malate the incubation mixture contained 3·0 ml. of 1:4 extract of cattle lens-cortex, 0·6 ml. of 0·1 M-malate, 0·2 ml. of 10 mM-$MnCl_2$, 0·3 ml. of 0·25% EDTA, 3 μmoles of NADP (0·3 ml.) and 0·1 ml. of 20 mM-H_2O_2. The control incubation mixture contained 3·0 ml. of 1:4 cattle lens-cortex extract, 0·2 ml. of 10 mM-$MnCl_2$, 0·3 ml. of 0·25% EDTA, 0·9 ml. of water and 0·1 ml. of 20 mM-H_2O_2. Tubes were left at room temperature. The H_2O_2 was added after zero-time samples had been taken. Then 0·5 ml. samples were taken at intervals. Protein was precipitated by addition of 1·5 ml. of 2·5% metaphosphoric acid and GSH was estimated in the supernatants by the method of Grunert & Phillips (1951).

Expt. no.	Time (min.)	Total GSH remaining (μmoles)	
		With malate	Control
1	0	9·4	8·8
	2	6·4	6·8
	30	8·2	7·2
	90	8·2	6·5
2	0	5·8	6·3
	2	3·9	3·6
	30	4·3	3·6
	60	4·8	3·0

Hydrogen peroxide in aqueous humour. Aqueous humour was removed from cattle eyes within 20 min. of death and kept in the dark on ice until examined approximately half an hour later. The aqueous humour was taken from anaesthetized rabbits and could be tested within 0·5 min. of its removal from the eye. Table 6 shows that traces of hydrogen peroxide are present in the aqueous humour of both cattle and rabbits. The oxidation of ascorbic acid in aqueous humour is accelerated by light (Pirie, 1965) and, as in milk (Hopkins, 1938), this appears to be a photo-oxidation catalysed by riboflavine. Philpot & Pirie (1943) found traces of riboflavine

Table 6. *Hydrogen peroxide in aqueous humour*

Reduction of 2,6-dichlorophenol-indophenol by ascorbic acid and its oxidation by H_2O_2 and peroxidase were measured spectrophotometrically at 600 mμ and pH 6·6, as described in the Methods section. Each cell contained 1·0 ml. of 0·1 M-phosphate buffer, pH 6·6, 2,6-dichlorophenol-indophenol (1·0 ml. ≡ 0·1 mg. of ascorbic acid) and water to 2·8 ml. The extinction at 600 mμ was read and a sample of aqueous humour was added such that an excess of dye remained. A reading of extinction was taken and peroxidase added by wetting the end of a polythene rod in the undiluted enzyme (Boehringer, 10 mg./ml.) and stirring the contents of the cell with it. A further reading was made. To another cell containing the same amounts of dye and aqueous humour, catalase was first added, followed 1 min. later by peroxidase and the final extinction was read.

Expt. no.	Species	Vol. of aqueous humour (ml.)	Time out of eye	Change in E_{600}: Aqueous humour	Aqueous humour + peroxidase	Aqueous humour + catalase + peroxidase
1	Cattle	0·15	1 hr. at 2°	−0·507	+0·011	0
2	Cattle	0·30	1 hr. at 2°	*	+0·022	+0·008
3	Cattle	0·30	1 hr. at 2°	*	+0·037	+0·013
4	Cattle	0·15	1 hr. at 2°	−0·778	+0·016	0
5	Cattle	0·1	1 hr. at 2°	−0·413	+0·017	—
6	Rabbit	0·08	25 sec.	−0·386	+0·008	—
	Rabbit	0·12	5 min.	−0·603	+0·016	—
7	Rabbit	0·05	1 min.	*	+0·016	+0·006
	Rabbit	0·05	5 min.	*	+0·020	+0·008
9	Cattle, 0·5 μg. of riboflavine added to 1·0 ml. of aqueous humour	0·1	0 min. after riboflavine	−0·360	+0·026	—
		0·1	6 min. after riboflavine	−0·200	+0·068	—
		0·1	15 min. after riboflavine	−0·150	+0·055	—

* Initial extinction of dye too high for measurement.

Table 7. *Effect of catalase on oxygen uptake and ascorbic acid oxidation in aqueous humour*

Cattle aqueous humour was taken 30 min. after death and kept on ice in the dark until used. Each Warburg flask contained 0·5 ml. of M-phosphate buffer, pH 7·4, or M-tris–HCl, pH 6·9, 2·0 ml. of aqueous humour and 0·5 ml. of water. Then 5 μl. of undiluted catalase (10 mg./ml.) was added to half the manometer flasks. The centre well contained KOH. Temperature was 30° and the gas phase was air. At the end of the experiment 1·0 ml. of 10% metaphosphoric acid was added to each manometer flask, and the fluids were chilled and centrifuged to remove the trace of protein in aqueous humour. Ascorbic acid was estimated in the supernatant by titration with 2,6-dichlorophenol-indophenol, as described in the Methods section.

Expt. no.	Time (min.)	O_2 uptake (μmoles): Without catalase	With catalase	Loss of ascorbic acid (μmoles): Without catalase	With catalase
1	110	1·65	0·76	2·1	1·3
2	60	1·0	0·5	Total (2·7)	2·4
3	65	2·0	1·0	Total (2·2)	1·5
4	120	2·3	1·4	Total (2·8)	2·0

in cattle aqueous humour. Expt. 9 of Table 6 shows that addition of 0·5 μg. of riboflavine to 1·0 ml. of aqueous humour causes rapid oxidation of ascorbic acid with formation of hydrogen peroxide. The hydrogen peroxide does not increase proportionally as the ascorbic acid becomes oxidized but this is probably due to its reaction with further ascorbic acid (Mapson, 1945).

The formation of hydrogen peroxide during oxidation of ascorbic acid in aqueous humour was also detected manometrically. The oxygen uptake of aqueous humour in the presence and absence of catalase was examined, and, just as with glucose and glucose oxidase, the addition of catalase approximately halved the oxygen uptake. Table 7 also shows that catalase diminished the total loss of ascorbic acid.

Diffusion of hydrogen peroxide into the lens. Pairs of cattle lenses taken from the same animal were incubated either in buffer at pH 7·4 or in this buffer

Table 8. *Diffusion of hydrogen peroxide into the lens*

Paired cattle lenses were incubated in 10ml. of Krebs phosphate buffer, pH 7·4; H_2O_2 (2mM) was added to the medium of one lens. The lenses were rocked in a water bath at 37° in air for 6 or 9hr. After incubation the lenses were removed from the medium and ground with 10ml. of 5% metaphosphoric acid. After being left at 2° overnight they were centrifuged and GSH was estimated in the supernatants by the method of Grunert & Phillips (1951). GSSG was estimated by the method of Bergmeyer (1963). In Expts. 3 and 4 glucose (2g./l.) was added to the medium of one of a pair of lenses and H_2O_2 (2mM) to the medium of both.

		GSH content (μmoles/lens)			GSSG content (μmoles/lens)		
Expt. no.	Time (hr.)	Lens	Lens+ H_2O_2	Change due to H_2O_2	Lens	Lens+ H_2O_2	Change due to H_2O_2
1	6	20·4	16·4	−4·0	0·3	1·6	+1·3
2	9	15·8	11·7	−4·1	0·5	1·4	+0·9
		Lens+ H_2O_2	Lens+ H_2O_2+ glucose	Change due to glucose	Lens+ H_2O_2	Lens+ H_2O_2+ glucose	Change due to glucose
3	6	13·2	16·8	+3·6	2·0	0·8	−1·2
4	9	11·1	15·8	+4·7	2·4	0·6	−1·8

to which hydrogen peroxide (2mM) had been added. After incubation, the lenses were removed and GSH and GSSG were determined. Table 8 shows that after 6–9hr. incubation at 37° in a medium containing hydrogen peroxide the GSH concentration of the lens fell and that of GSSG increased. The lens therefore appears to be permeable to hydrogen peroxide. Expts. 3 and 4 of Table 8 show that if glucose, as well as hydrogen peroxide, is added to the medium, the fall in GSH concentration is diminished and so also is the rise in that of GSSG. It appears likely that the glucose 6-phosphate dehydrogenase of lens is supplying $NADPH_2$ to lens glutathione reductase.

Coupling of ascorbic acid oxidation with glutathione peroxidase. The following reactions are known to occur:

$$\text{Ascorbic acid} + O_2 \rightarrow \text{dehydro-L-ascorbic acid} + H_2O_2$$

$$\text{Ascorbic acid} + H_2O_2 \rightarrow \text{dehydro-L-ascorbic acid} + 2H_2O$$

$$2GSH + \text{dehydro-L-ascorbic acid} \rightarrow GSSG + \text{ascorbic acid}$$

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

The first three reactions are not known to be enzymic in animal tissues; the fourth can be both non-enzymic and catalysed by glutathione peroxidase. Experiments were therefore set up to determine whether glutathione peroxidase of lens reacts with hydrogen peroxide formed during ascorbic acid oxidation. To allow for the non-enzymic reaction between GSH and dehydro-L-ascorbic acid a control was set up containing both GSH and ascorbic acid but without lens, and, to allow as far as possible for any non-specific effect of protein, a control was set up in which the lens extract was boiled before being added to the other reagents.

Table 9 shows that, in a mixture of ascorbic acid and glutathione in air, more glutathione will disappear in the presence of lens (glutathione peroxidase) than in its absence, the enzyme competing with ascorbic acid for the available hydrogen peroxide. The non-enzymic removal of GSH during the oxidation of ascorbic acid is considerable, but the addition of an extract of lens accelerates the removal of GSH. A boiled lens extract does not accelerate removal of GSH. The same result was obtained if lens extract was mixed with aqueous humour together with extra ascorbic acid and GSH (Table 9). Though the addition of a lens extract increased the removal of GSH it diminished the removal of ascorbic acid. The greater loss of ascorbic acid in the buffer control compared with loss in the boiled enzyme control may be due to combination of trace metals with lens proteins.

DISCUSSION

Evidence given in the present paper shows that glutathione peroxidase is present in lens. Traces of hydrogen peroxide are present in aqueous humour and it is formed during oxidation of the contained ascorbic acid. This oxidation is, *in vitro*, catalysed by light, and it seems probable that riboflavine is the catalyst (Pirie, 1965). Philpot & Pirie (1943) found traces of riboflavine or FAD (or both) in aqueous humour. Here therefore is a situation unique to the eye arising from the low activity of catalase in the lens and the presence of a light-catalysed

Table 9. *Effect of lens on changes in glutathione content during oxidation of ascorbic acid in aqueous humour or in phosphate buffer*, pH 7·4

The lens–aqueous humour mixture contained 3·0 ml. of 1:4 extract of cattle lens-cortex, 8·0 ml. of cattle aqueous humour and 1·0 ml. of 1·0 M-phosphate buffer, pH 7·4. Boiled lens: a mixture of 5·0 ml. of lens–aqueous humour and 0·5 ml. of 0·25% EDTA was immersed in a boiling-water bath for 3 min. and cooled to 20°; then 0·2 ml. of 0·1 M-GSH, 0·3 ml. of 0·1 M-ascorbic acid and 2·5 μg. of riboflavine (0·05 ml.) were added to complete the incubation mixture.

Unboiled lens: the incubation mixture was as above but the lens–aqueous humour was not boiled.

Control without lens: 4·0 ml. of aqueous humour, 1·5 ml. of water and 0·5 ml. of 1·0 M-phosphate buffer, pH 7·4, were mixed; the incubation mixture contained 5·0 ml. of the above mixture, 0·5 ml. of 0·25% EDTA, 0·2 ml. of 0·1 M-GSH, 0·3 ml. of 0·1 M-ascorbic acid and 2·5 μg. of riboflavine (0·05 ml.).

The flasks were placed in open sunshine at 20°. For estimation of GSH, 1·0 ml. samples were added to 2·0 ml. of 6% metaphosphoric acid, chilled and left at 4° for at least 30 min. They were then centrifuged and the GSH was estimated in the supernatant by the method of Grunert & Phillips (1951). In Expt. 2 the lens was diluted with 0·1 M-phosphate buffer, pH 7·4, instead of with aqueous humour. Ascorbic acid was estimated as described in the Methods section.

		Loss of GSH (μmoles/ml. of incubation mixture)			Loss of ascorbic acid (μmoles/ml. of incubation mixture)		
Expt. no.	Time (min.)	Boiled lens	Lens	Control without lens	Boiled lens	Lens	Control without lens
1	15	0·29	0·81	0·32	—	—	—
	45	0·83	1·60	0·95	—	—	—
	100	1·86	2·64	1·88	0·84	0·63	1·43
2	15	0·12	0·54	0·24	0·29	0	0·34
	45	0·49	1·22	0·56	—	—	—
	100	0·98	2·06	1·13	1·03	0·76	2·04

reaction forming hydrogen peroxide in the aqueous humour.

The question of enzymic participation in the removal of hydrogen peroxide formed in the oxidation of ascorbic acid is complicated by the fact that ascorbic acid, dehydro-L-ascorbic acid, GSH and hydrogen peroxide react non-enzymically at physiological pH. But Table 9 shows that the addition of an extract of lens accelerates the removal of GSH during the oxidation of ascorbic acid. It is therefore possible that the respiratory link between ascorbic acid and glutathione, which has been so long sought (Kinoshita, 1964), is not oxygen but hydrogen peroxide. This link provides GSSG, which, by oxidizing $NADPH_2$, makes NADP available for the enzymes of the pentose phosphate pathway, the malic enzyme and others. Thus the oxidation of ascorbic acid in aqueous humour could in this way be linked with the metabolism of glucose and other substances in lens.

Hydrogen peroxide may also be generated by enzymic reactions within the lens. Such enzymes as aldehyde oxidase, monoamine oxidase, diamine oxidase, L-amino acid oxidase, D-amino acid oxidase and xanthine oxidase produce hydrogen peroxide. Jocelyn (1964) finds that hydrogen peroxide, formed by xanthine oxidase in liver, accounts for the oxidation of GSH in that tissue. Gillette, Brodie & La Du (1957) have described an enzyme in rat-liver microsomes that oxidizes the reduced coenzymes aerobically with the production of hydrogen peroxide. A similar enzyme has been described in leucocytes by Iyer, Islam & Quastel (1961) and Roberts & Quastel (1964). These hydrogen peroxide-producing enzymes have not been described in lens but nevertheless may be present.

Although it is suggested in the present paper that hydrogen peroxide is a respiratory link, excess can be toxic. Cohen & Hochstein (1964) find that hydrogen peroxide is a toxic intermediate common to many of the haemolytic agents. Naphthalene, which is haemolytic, is also cataractogenic, and the loss of GSH from the lens which is a general feature of cataract formation is particularly swift after feeding with naphthalene. The question of hydrogen peroxide formation by cataractogenic substances needs study.

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