Cataract Produced by Tyrosinase and Tyrosine Systems in Rabbit Lens *in vitro*

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1. Cataracts were developed by incubating rabbit lenses for 22 hr. at 37° in a culture medium containing tyrosine and tyrosinase (EC 1.10.3.1). 2. A 45% diminution in the content of GSH and significant inhibition of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) activity were observed in the cataractous lenses compared with controls. 3. GSSG accumulated in both cataractous and control lenses. Significant amounts of GSSG were transported outward from the cataractous lenses and small amounts from control lenses. 4. Transport of GSSG from rabbit lens incubated in a diffusate of plasma from a naphthalene-fed rabbit was also observed. 5. GSSG was found in the aqueous humour obtained between 2 and 24 hr. after feeding of naphthalene to rabbits; subsequently the GSSG in the aqueous humour decreased to almost undetectable amounts in 48 hr.; in controls, GSSG was not detectable. 6. A possible mechanism of formation of experimental and senile cataract is briefly discussed.

Ogino & Yasukura (1957) first suggested that quinonoids might be the cataractogenic substances in senile cataract and in cataracts induced by naphthalene, tyrosine, galactose, dinitrophenol and alloxan-diabetes. Quinonoids have not been implicated in diabetic cataract. The only cataract in which a quinone has definitely been shown to be the toxic compound is naphthalene cataract. van Heyningen & Pirie (1967) and Rees & Pirie (1967) found that 1,2-dihydroxynaphthalene, a metabolic product of naphthalene, is rapidly autoxidized to 1,2-naphthaquinone, which may react with various constituents of the lens and oxidize them.

Although the mechanism of development of senile cataract and cataracts formed through the action of various toxins is not clearly understood, it is possible that oxidizing substances to which lens capsule is permeable may appear in the aqueous humour and that these substances may oxidize any NADPH, GSH and protein thiol within the lens. These changes would ultimately lead to precipitation of lens protein and to opalescence (Kinoshita, 1964).

One physiologically occurring substance that might play a role in cataractogenesis is dopaquinone,* which can be formed from tyrosine during its oxidation by tyrosinase (EC 1.10.3.1) present in the ciliary processes and the iris (Bowness & Morton, 1953). In the present study we have investigated the possible role of tyrosine oxidation

in cataract formation. Bernheimer (1964) demonstrated the presence of dopa and catecholamines in calf iris. Tyrosinase was shown to be present in active form in senile cataractous lens (Srivastava & Nath, 1968) and we demonstrated that the oxidation product of tyrosine could produce cataract in rabbit lens *in vitro* (Srivastava & Beutler, 1968a). Subsequently, Pirie (1968) showed that the oxidation products of tyrosine can combine with bovine lens protein to give a brown protein, which is similar in its fluorescence spectrum to the brown protein found in naphthalene cataract in rabbits and in some human cataracts. These observations suggest that dopaquinone could play a direct role in the origin of at least some human cataracts. Another mechanism for the oxidation of tyrosine may be by the iron-ascorbic acid-EDTA system (Lissitzky, Rolland, Reynaud & Lasry, 1962), since tyrosine, ascorbic acid and iron are present in the lens (van Heyningen, 1968). The dopa formed is readily autoxidized to dopaquinone.

MATERIALS AND METHODS

Reagents. Tyrosinase from mushroom (500–600 enzyme units/mg.), NAD⁺, NADP⁺, NADPH, GSH, GSSG and glutathione reductase (EC 1.6.4.2) (100 enzyme units/ml.) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Tyrosine was purchased from Calbiochem, Los Angeles, Calif., U.S.A.

Production of cataract by tyrosine-tyrosinase system *in vitro*. The culture medium was prepared with a slight modification of the method described by Kinoshita, Merola

* Abbreviation: dopa, 3,4-dihydroxyphenylalanine.

& Hayman (1965). The final composition of the culture medium was: MgSO_4 , 0.46mM; Na_2HPO_4 , 0.24mM; KH_2PO_4 , 0.25mM; KCl , 3.33mM; NaHCO_3 , 23.1mM; KHCO_3 , 0.84mM; glucose, 5.0mM; NaCl , 129mM; CaCl_2 , 1.25mM. The CaCl_2 solution was prepared separately and added to the medium at the time of lens culture. The medium was sterilized by filtering through Millipore filters (obtained from Millipore Filter Corp., Bedford, Mass., U.S.A.). Penicillin and streptomycin were added at a concentration of 0.6ml./100ml. of medium (penicillin, 10000 i.u./ml., and dihydrostreptomycin, 10mg./ml.). A 10ml. sample of culture medium was transferred to a culture tube under sterile conditions, and oxygen was bubbled through the solution for 5 min. To study the effect of the tyrosine-tyrosinase system in cataract formation, 1mg. of tyrosine and 300 enzyme units of tyrosinase were added.

Rabbits weighing 2.0–2.5 kg., about 6 months old, were killed by injecting air into a marginal ear vein. Whole eyes were excised and the lenses, with intact capsules, were removed by a posterior approach. The lenses were washed with the culture medium and blotted immediately with moist filter paper. One of a pair of lenses was placed in the medium containing tyrosine and tyrosinase (mushroom), designated 'experimental', and the other was placed in the medium without tyrosine and tyrosinase, designated 'control'. The lenses were then incubated for 22 hr. at 37° under sterile conditions.

Naphthalene-fed rabbits. One group of 30 rabbits was given 2.0 g. of naphthalene/kg. body wt., administered as 10% (w/v) naphthalene in edible Crisco oil, by using a gastric tube. The control group of eight rabbits was given only edible Crisco oil. At intervals of 2, 4, 8, 24 or 48 hr. after the feeding of naphthalene, aqueous humour from the anterior chamber was collected from one or both eyes under light ether anaesthesia and analysed for GSSG content. Aqueous humour from the same eye was never sampled twice.

Incubation of the lens in a diffusate of plasma from a naphthalene-fed rabbit. Naphthalene dissolved in edible Crisco oil (2.0 g./kg. body wt.) was given to a rabbit. A control rabbit was given an equivalent amount of edible Crisco oil. At 3 hr. after feeding, blood was collected in heparin, and plasma was separated. A 10ml. sample of plasma from the experimental animal and a similar sample from the control animal were dialysed for 7 days at 4° against 200 ml. of water containing a few drops of chloroform (van Heyningen & Pirie, 1967). The diffusate was concentrated to 10ml. in a rotary-flask evaporator. To each concentrate 0.12 ml. of 10% (w/v) glucose and 1.2 ml. of 0.1 M-potassium phosphate buffer, pH 7.4, were added. One of a pair of rabbit lenses was incubated at 37° in each medium for 22 hr.

Thiol and protein determinations. A 10% homogenate of lens was made in phosphate-saline, pH 7.4 (1 part of 0.15 M-potassium phosphate buffer, pH 7.4, plus 9 parts of 0.15 M- NaCl), in a Potter-Elvehjem glass homogenizer, and samples were taken for the determination of GSH and of total thiol (protein thiol and non-protein thiol) (Beutler, Duron & Kelly, 1963; Ellman, 1959), with 5,5'-dithiobis-(2-nitrobenzoic acid). GSSG in the lens homogenate, culture medium and aqueous humour was determined enzymically with glutathione reductase [NAD(P)H -glutathione oxidoreductase, EC 1.6.4.2] and NADPH, after

alkylation of GSH with *N*-ethylmaleimide (Srivastava & Beutler, 1968b). Protein was determined by the biuret method (Gornall, Bardawill & David, 1949).

Enzyme assays. The 10% lens homogenate was centrifuged at 10000g for 10 min. at 4°, and the supernatant was used for enzyme assay. All assays were carried out in a Gilford model 2000 multiple-sample recording spectrophotometer at 25° with a light-path of 1.00 cm. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.43) activities were assayed by a modification of the method of Glock & McLean (1953), with 0.05 ml. samples of the supernatant. The final concentrations in a 1.0 ml. system were: MgCl_2 , 10mM; tris-HCl buffer, pH 8, 100mM; NADP^+ , 0.1mM; glucose 6-phosphate or 6-phosphogluconate, 0.6mM. Cuvette no. 1 contained all the components except the substrate; cuvette no. 2 contained both glucose 6-phosphate and 6-phosphogluconate, and cuvette no. 3 contained only 6-phosphogluconate. For calculation of the glucose 6-phosphate dehydrogenase activity the change in E_{340} in cuvette no. 3 was subtracted from the change in E_{340} in cuvette no. 2. Lactate dehydrogenase (EC 1.1.1.27) activity was assayed by the method of Neilands (1955), with 0.01 ml. of supernatant. One unit of enzyme effected the reduction of 1 nmole of NADP^+ or NAD^+ /min. at 25°.

RESULTS

Cataract produced by tyrosine-tyrosinase system in rabbits

Morphological changes. The lens incubated in the culture medium to which tyrosinase and tyrosine had been added became cloudy and slightly opalescent with a brownish cast. However, the nucleus remained clear. The lens capsule in the cataractous lens turned brownish and the browning appeared to be extended throughout the cortex. The control lens remained clear and transparent. The mean $\pm$ S.E.M. wet weights of the experimental and control lenses were 374 ± 8.3 mg. and 361 ± 10.4 mg. respectively. In most cases the weight of the experimental and control lenses was exactly the same; however, in a few cases the weight of the experimental lens was slightly more than that of the control.

GSH, GSSG and total thiol. The GSH content in the cataractous lens was lowered by about 50% as compared with that of the control when expressed per g. fresh wt. and 44% when expressed per lens (Table 1). Small amounts of GSSG were found in both experimental and control lenses. There was no measurable amount of GSH in the culture medium of experimental or control lenses. However, GSSG was found in the culture medium of both experimental and control lenses; the concentration of GSSG in the culture medium of the experimental lens was always three to five times that in the control medium (Table 1). The total thiol content was approx. 27% lower in the experimental than in the control lenses. The protein

Table 1. *GSH, GSSG and protein thiol in cataractous rabbit lens in vitro and in medium of incubation*

All the values of GSH, GSSG, total thiol and protein thiol are means $\pm$ s.e.m. of 16 rabbit lenses after incubation. One lens from each rabbit was incubated in the control medium; the other lens was incubated in the medium containing tyrosine and tyrosinase. Protein thiol was calculated by subtracting GSH from the total thiol. For experimental details, see the Materials and Methods section.

Group	Addition to medium	GSH			GSSG			Total thiol			Protein thiol		
		(nmoles/100 mg. fresh wt.)	(nmoles/lens)	(nmoles/10 ml. of medium)	(nmoles/lens)	(nmoles/10 ml. of medium)	(nmoles/100 mg. fresh wt.)	(nmoles/lens)	(nmoles/100 mg. fresh wt.)	(nmoles/lens)	(nmoles/100 mg. fresh wt.)	(nmoles/lens)	(nmoles/100 mg. fresh wt.)
Control	Nil	850 ± 60	3017 ± 220	0	18.5 ± 3.2	71.0 ± 11.6	3575 ± 138	12515 ± 436	2716 ± 103	9498 ± 293	2716 ± 103	9498 ± 293	2716 ± 103
Experimental	Tyrosine + tyrosinase	451 ± 61	1676 ± 231	0	81.0 ± 19.2	288.0 ± 32.2	1831 ± 121	10486 ± 492	2381 ± 72	8810 ± 318	2381 ± 72	8810 ± 318	2381 ± 72

thiol, calculated by subtracting GSH from the total thiol, was slightly lowered (about 20%) in experimental lenses. In some experiments, 1.0 mg. of tyrosine was also added to the control culture medium, and the results were comparable with the controls without tyrosine. However, if 300 enzyme units of tyrosinase were added to the control culture medium, it brought about slight diminution in non-protein thiol content.

Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The activity of glucose 6-dehydrogenase in the cataractous lenses compared with that in the control lenses was decreased by about 45% when expressed per g. of lens and 32% when expressed per mg. of protein (Table 2), whereas 6-phosphogluconate dehydrogenase activity was not markedly affected.

Lactate dehydrogenase. The activity of lactate dehydrogenase in the experimental lenses compared with that in the control lenses was decreased by about 30% when expressed per 100 mg. of lens and about 22% when expressed per mg. of protein (Table 2).

Outward transport of GSSG from the lens of naphthalene-treated rabbits

At 2 hr. after naphthalene had been given to rabbits, considerable amounts of GSSG were detected in the aqueous humour obtained from the anterior chamber (Fig. 1). The amount of GSSG in the aqueous humour remained elevated until 24 hr. after naphthalene administration and then decreased to almost an undetectable value after 48 hr. No GSSG was detected in the aqueous humour of control rabbits given only the edible oil used for dissolving naphthalene.

Outward transport of GSSG from the lens incubated in a diffusate of plasma from a naphthalene-treated rabbit

After 22 hr. of incubation of the lens in the experimental medium containing diffusate of plasma from a naphthalene-treated rabbit, the lens turned pale brown; the lens in the control medium remained clear and colourless, as shown by van Heyningen & Pirie (1967). The concentration of GSH in the experimental lens was 40% of that in the control lens. Very small amounts of GSSG (< 10 nmoles) were detected in the control lens and medium, whereas in the experimental lens and the total medium respectively 150 and 188 nmoles of GSSG were found. Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and lactate dehydrogenase activities were comparable in experimental and control lenses.

Table 2. *Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and lactate dehydrogenase activities in cataractous rabbit lens in vitro*

The values are means $\pm$ s.e.m. of 16 rabbit lenses. One lens from each rabbit was incubated in the control medium; the other lens was incubated in the medium containing tyrosine and tyrosinase. One unit of glucose 6-phosphate dehydrogenase or of 6-phosphogluconate dehydrogenase reduced 1 nmole of NADP⁺/min. at 25°. One unit of lactate dehydrogenase reduced 1 nmole of NAD⁺/min. at 25°.

Group	Addition to medium	Glucose 6-phosphate dehydrogenase activity			6-Phosphogluconate dehydrogenase activity			10 ⁻² $\times$ Lactate dehydrogenase activity		
		(units/100 mg. fresh wt.)	(units/mg. of protein)	(units/mg. of fresh wt.)	(units/100 mg. fresh wt.)	(units/mg. of protein)	(units/mg. of fresh wt.)	(units/100 mg. fresh wt.)	(units/mg. of protein)	(units/mg. of fresh wt.)
Control	Nil	12.06 $\pm$ 1.08	0.397 $\pm$ 0.029	24.28 $\pm$ 1.98	0.811 $\pm$ 0.058	6.85 $\pm$ 1.30	0.22 $\pm$ 0.04	6.85 $\pm$ 1.30	0.22 $\pm$ 0.04	0.22 $\pm$ 0.04
	Tyrosinase + tyrosine	6.75 $\pm$ 0.72	0.250 $\pm$ 0.024	20.21 $\pm$ 1.45	0.755 $\pm$ 0.052	4.86 $\pm$ 0.86	0.17 $\pm$ 0.03	4.86 $\pm$ 0.86	0.17 $\pm$ 0.03	0.17 $\pm$ 0.03

Fig. 1. Concentration of GSSG in the aqueous humour after naphthalene had been given to rabbits. The numbers in parentheses represent the numbers of samples of aqueous humour; other conditions are given in the text. The vertical bars indicate $\pm$ 1 s.e.m.

DISCUSSION

It is possible to develop lens opacity by incubation of the rabbit lens in a culture medium containing tyrosine and tyrosinase. The pattern of biochemical alterations in the cataractous lens are similar to those observed in senile cataract in the human or in experimental cataract produced by cataractogenic substances. Glucose 6-phosphate dehydrogenase activity was significantly decreased in the cataractous lens, whereas the activity of 6-phosphogluconate dehydrogenase was not significantly affected. The content of GSH was significantly lowered in cataractous lens. In earlier studies we showed that the lens is permeable to GSSG but not to GSH (Srivastava & Beutler, 1968a), and that the permeability of the lens is unidirectional. It is possible that the transport of GSSG from the lens is an active process like the outward transport of GSSG from erythrocytes (Srivastava & Beutler, 1968c, 1969) since it occurs against a concentration gradient in lens (S. K. Srivastava & E. Beutler, unpublished work) as well as in erythrocytes (Srivastava & Beutler, 1969). Normally no GSSG is detectable in the aqueous humour even when a very sensitive method is used for the determination. However, when naphthalene is given to rabbits appreciable amounts of GSSG are found in the aqueous humour. van Heyningen & Pirie (1967) and Rees & Pirie (1967) showed that, on giving naphthalene to rabbits, substances capable of oxidizing NADPH, GSH and protein thiol appear in the aqueous humour; thus oxidizing substances in aqueous humour to which the lens capsule is permeable may oxidize NADPH, GSH and protein

thiol in the lens. The oxidation of GSH increases the GSSG content of the lens, and the GSSG is transported out of the lens and is detectable in appreciable amounts in the aqueous humour (Fig. 1).

The incubation of the lens in a diffusate of plasma from a naphthalene-fed rabbit confirmed the observations by van Heyningen & Pirie (1967), and further demonstrated that GSSG is transported from the lens to the medium of incubation.

The production of cataracts and associated biochemical changes in lenses incubated in the tyrosine-tyrosinase system suggests a possible role of this system in cataract formation. The elaboration of the cell membranes of the ciliary epithelium was shown to be associated with the production of aqueous humour (Pappas, Smelser & Brandt, 1959). Ciliary processes and iris are known to contain high activities of tyrosinase (Bowness & Morton, 1953). When only tyrosine is present as substrate for this enzyme, there is a lag period of 3–5 min. before dopa is formed and before it can be converted into dopaquinone (Adachi & Halprin, 1967). In the presence of dopaquinone, however, the lag period does not occur, and transformation of tyrosine into dopaquinone proceeds quickly. The normal rate of flow of aqueous humour may be sufficiently high to prevent immediate conversion of tyrosine into dopa and dopaquinone by continual removal of traces of dopaquinone that might be formed. With advancing age, and particularly with senile cataract, the rate of flow of aqueous humour is diminished (Linner, 1959). It is possible that under these circumstances sufficient quantities of dopaquinone may accumulate in the ciliary processes to permit immediate conversion of tyrosine into more dopaquinone. If this powerful oxidizing agent reaches the lens through the aqueous humour, oxidation of NADPH, GSH or protein thiol would be expected, and GSSG, to which the lens is permeable, would appear in the aqueous humour. Once the amounts of thiols and ascorbic acid, which are potent inhibitors of tyrosinase, are decreased sufficiently, tyrosinase becomes active in the lens (Srivastava & Nath, 1968). The presence of tyrosine and tyrosinase in the lens would lead to the production in the lens of excess of dopaquinone, which could then

combine with the lens proteins. Such a course of events might explain some of the known biochemical findings about the formation of some types of cataracts.

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