Biochemical and Clinical Changes in the Rabbit Lens during Alloxan Diabetes

By J. W. WATERS

Nuffield Laboratory of Ophthalmology, University of Oxford

(Received 3 December 1949)

The nature of the chemical changes underlying the formation of lens opacities is still not clear. Much work has been done on human cataractous lenses, but analysis of these can only describe the end result of processes leading to cataract, because no lens is removed from a human eye until opacity is complete. Production of experimental cataract in animals has made possible the study of the chemistry and metabolism of the lens in the pre- and early cataractous stages, and such studies may show what chemical changes precede or accompany opacity formation. Bellows (1944) gives a summary of the work already done in this field. It has been established (e.g. Reis, 1912; Shoji, 1927, 1931; Cohen, Kamner & Killian, 1927; Gifford, 1932; Bellows & Rosner, 1937; Rosner, Farmer & Bellows, 1938) that the fully cataractous lens gives a much reduced reaction with sodium nitroprusside. This has been attributed to a decrease in glutathione content which in the normal rabbit lens averages 320 mg./100 g. (Bellows, 1944), equivalent to 34.6 mg. sulphur/100 g. The normal eye also contains a high concentration of ascorbic acid within the fluids and tissues. The high concentration of glutathione and ascorbic acid make it possible that these substances are concerned with the metabolism of the lens, and it seemed interesting to determine whether they are reduced in amount early in cataract formation or whether there is no change until the opacity is well advanced.

Cataracts produced in rabbits made diabetic with alloxan were used as experimental material. Dunn, Sheehan & McLetchie (1943), following the earlier work of Jacobs (1937), found that intravenous injection of alloxan produced permanent diabetes in animals. The pathology of alloxan diabetes has been fully discussed by Bell (1948) and by Lukens (1948). Bailey, Bailey & Leach (1944) first reported cataract in such animals and this was confirmed by Duffy (1945). Both authors found that cataract did not occur in every diabetic animal. This is borne out in my experiments. The cataract which occurs in alloxan diabetes closely resembles the type of cataract seen in young human diabetics. It therefore seemed reasonable to use alloxan-induced cataract in spite of the uncertainty with which it appears in diabetic rabbits.

METHODS

Induction of diabetes. Normal young adult rabbits, mostly small Dutch, were given intravenous injections of alloxan in doses of 175–200 mg./kg. body weight. The alloxan, dissolved in about 10 ml. sterile water, was injected slowly into the marginal ear vein in a single dose spread over a period of 5 min. or more. Since the injection appeared painful the vein in the neighbourhood of the injection site was anaesthetized by subcutaneous injection of procaine hydrochloride 5% (w/v) in liq. adrenal. 1/100,000. Each animal was closely watched during the 60 hr. following injection. The animals usually developed signs of hypoglycaemia 4–10 hr. after injection of alloxan, when 50% (w/v) glucose solution was administered, if necessary intravenously, but usually by stomach tube, to prevent convulsions. A high blood sugar usually developed 24–48 hr. later, although there was considerable variation. The hyperglycaemia was controlled for the first few days by intramuscular injection of insulin (20 units/ml.); 1–4 units/day were generally required. Occasionally a rabbit developed lipaemia. This was noticed by a 'cream' appearing on the surface of blood taken for sugar estimation; attempts were made to control it by giving balanced injections of glucose and insulin, but in only one case could the lipaemia be cured; most of these animals died within 3 or 4 days. All animals were examined often during the experiment and the blood-sugar level closely followed. The eye condition was studied by slit-lamp examination and by ophthalmoscopy after dilatation of the pupil with atropine. In three animals iridectomy was performed in order to see the periphery of the lens more clearly.

Blood sugar. The sugar content of blood taken from the marginal ear vein was determined by the ferricyanide method (Kramer & Steiner, 1931; Hagedorn & Jensen, 1923).

Ascorbic acid. Ascorbic acid in the aqueous humour and lens was determined by titration with 2:6-dichlorophenol-indophenol, using the method of Bessey & King (1933). Aqueous humour was removed with a fine capillary pipette immediately after death, but before the eye was removed, and transferred to a centrifuge tube containing 0.2 ml. of 30% metaphosphoric acid. The volume was made up to 3 ml. with distilled water. Any precipitate was centrifuged off and the ascorbic acid in the supernatant liquid titrated against the dye. The lens, without capsule, was removed from the eye, weighed, dropped into a mortar containing 1 ml. 30% metaphosphoric acid and ground to a paste; 1 ml. distilled water was added and grinding continued until the mixture was homogeneous. The suspension was washed into a centrifuge tube with two 1 ml. portions of

distilled water. After centrifuging, the ascorbic acid in the supernatant fluid was titrated.

Thiol sulphur. Thiol S is present in the lens as the —SH groups of glutathione and of the lens proteins. The —SH content was determined in both fractions.

Glutathione. The glutathione —SH in a portion of the supernatant fluid used for the ascorbic acid estimation was determined by titration against 0.005 N- I_2 in KI solution by the method of Perlzweig & Delrue (1927). The I_2 value of the ascorbic acid present was calculated from the dye titration and subtracted from the total I_2 titration figure.

Total —SH. Chloroacetophenone (CAP) was used to estimate the —SH groups in whole lens solution, as described by Dixon (1948). Whole lens, without capsule, was weighed, ground with water to form an emulsion and made up to 5 ml. The —SH content of this solution was determined by reaction with CAP in Warburg manometers. To each cup was added 0.5 ml. of 1.3% (w/v) $NaHCO_3$ solution and 1.5 ml. water and, in the side arm, 0.02 ml. of 10% (w/v) solution of CAP in ethylene glycol monoethyl ether. In addition, 1 ml. of lens solution was added to the liquid in the body of the flask and the apparatus filled with N_2 containing 5% (v/v) CO_2 in the usual way. After equilibration the taps were closed and the reagents mixed. One mol. CO_2 is liberated for each g.equiv. of —SH present. The manometers were read after 10 min., by which time the reaction was complete. Controls containing lens solution alone and CAP alone were run at the same time.

Alloxan. This was determined using the method described by Archibald (1945) in which the alloxan is coupled with *o*-phenylene diamine and the fluorescence of the resulting compound measured.

RESULTS

Clinical. The general pattern of the results is summarized in Table 1. In the short-term experiments the animals were killed within 4 hr. of the completion of the alloxan injection. No clinical change in the eyes was seen during this time.

had the appearance of dust on a glass plate. It could be seen quite clearly by reflected light and occasionally rather indistinct radial spokes could be seen. The second stage was a more slowly developing opacity which had a chalky appearance. This dense white opacity could be seen with the unaided eye. In three animals vacuoles and irregular water clefts could be seen, especially where an iridectomy had been performed. The opacities were always bilateral, were only seen at the posterior pole, and never covered more than one-half the area of the lens. There was some discontinuity of refractive index below the anterior surface of the lens, although this did not amount to an opacity.

Two animals with normal blood-sugar values developed retinal degeneration.

A general pathological examination was made of one animal in which the blood-sugar value exceeded 700 mg./100 ml. for 6 days before death. This confirmed the fact that the main pathological change is in the pancreas where characteristic degeneration of the β -cells was found with no change in the acinar tissue.

Estimation of ascorbic acid and glutathione. The results of analysis of the aqueous humour and lens are set out in Tables 2 and 3. Table 2 shows that the ascorbic acid content of the aqueous humour and of the lens did not change during the experiment. The values show a wide scatter. Table 3 summarizes the analysis of the glutathione and protein thiol groups. The glutathione content of the lens after injection of alloxan is very variable, but in no case differs significantly from normal. On the other hand, the total —SH content of the lens decreased immediately after alloxan injection, and did not regain the normal value during the whole period of the experiment. Some rabbits lived 2 years after alloxan injection and

Table 1. *Summary of general results of alloxan treatment*

37 Rabbits treated with alloxan parenterally					
14 died within 1 week; normal eyes		8 killed within 4 hr. (short-term experiments)		15 lived more than 1 week	
7 with high blood sugar		10 with high blood sugar		5 with normal blood sugar	
7 with normal blood sugar		5 with normal eyes		3 with normal eyes	
3 with lipaemia		5 with bilateral cataract		2 with retinal degeneration	
4 without lipaemia					

Five of the surviving rabbits with blood-sugar values exceeding 300 mg. glucose/100 ml. developed bilateral posterior polar cataract 4–6 weeks after alloxan treatment. The first sign appeared as a slight 'furring' of the suture lines on the posterior pole of the lens. This opacity gradually spread in two definite stages. The first, more rapidly moving veil

the —SH content of the lens was still low when they were killed. Since the glutathione content appeared to remain normal this decrease in —SH must be due to a decrease in the thiol groups of the lens proteins. The last column of Table 3 gives the values for 'protein —SH' obtained by subtracting the mean value for the total —SH.

Table 2. *Ascorbic acid content of normal and alloxan-treated rabbit lens and aqueous humour*(Figures given in order are: Mean $\pm$ S.D. (extreme values), number of observations.)

	Aqueous humour (mg./100 ml.)	Lens (mg./100 ml.)
Normal	34.3 $\pm$ 3.6 (28.6-40.6) 8	12.1 $\pm$ 4.2 (6.9-19.0) 9
<4 hr. after alloxan; short-term experiments	36.2 $\pm$ 10 (23.5-50) 7	11.2 $\pm$ 3.3 (6.9-18.5) 11
>2 weeks after alloxan; high blood sugar	37.7 $\pm$ 10 (22-54) 5	9.0 $\pm$ 2.6 (5.3-10.8) 5
Bilateral cataract; high blood sugar	36.7 $\pm$ 13 (22-54.5) 5	8.1 $\pm$ 2.2 (5.3-10.7) 3

Table 3. *Thiol content of the lens of rabbits treated with alloxan*(Figures given in order are: Mean $\pm$ S.D. (extreme values), number of observations. Values for 'protein —SH' obtained by subtracting glutathione —SH from total —SH.)

	Glutathione (iodine) (mg. S/100 g. lens)	Total —SH (CAP) (mg. S/100 g. lens)	'Protein —SH' (mg. S/100 g. lens)
Normal	34.0 $\pm$ 7 (27-43) 3	187 $\pm$ 9.2 (173-195) 6	153
<4 hr. after alloxan; short-term experiments	55 $\pm$ 20 (19-95) 11	85 $\pm$ 19 (62-110) 8	30
>2 weeks after alloxan; normal blood sugar	35 $\pm$ 3 (30-43) 3	115 $\pm$ 9 (108-128) 3	80
>2 weeks after alloxan; high blood sugar	34.0 $\pm$ 7 (29-40) 5	101 $\pm$ 10 (93-115) 5	67
Bilateral cataract; high blood sugar	28 $\pm$ 3 (25-32) 3	108 $\pm$ 7.4 (98-115) 3	80

Effect of precipitant. Pirie (1946) found that oxidized glutathione present in solution with dialysed protein became reduced when treated with certain precipitating agents such as trichloroacetic acid. Metaphosphoric acid, however, seemed to be free from this effect. I have confirmed this using cystine. Lens solution dialysed to remove glutathione was mixed with a small quantity of cystine and the protein precipitated with 30% (w/v) metaphosphoric acid. The supernatant fluid after centrifuging gave a negative nitroprusside reaction. Application of the CAP method to this solution was made difficult by the buffering action of the phosphate. Removal as the barium salt gave an unbuffered solution which appeared not to react with CAP. The effect of precipitating agents on the state of glutathione is being investigated further.

Fate of alloxan. Alloxan disappeared from the blood and from the aqueous humour during the inevitable but short interval between the completion of the injection and removal of the fluids. Less than 2 μ g. was found in 0.2 ml. samples of either fluid, whereas, had the alloxan distributed itself evenly throughout the body and not reacted with the tissues, the samples would have contained at least 25-30 μ g.

DISCUSSION

Two main oxidation-reduction systems involving thiol groups are thought to be present in the lens. Bellows (1944) suggests that hydrogen from a sub-

strate is transferred to the sulphur of the protein; this in turn transfers hydrogen to glutathione and thence to dehydroascorbic acid. The reactions may be represented as follows:

- (1) $\text{RH}_2 + \text{PSSP} \rightleftharpoons 2\text{PSH} + \text{R}$,
- (2) $\text{GSSG} + 2\text{PSH} \rightleftharpoons \text{PSSP} + 2\text{GSH}$,
- (3) $2\text{GSH} + \text{dehydroascorbic acid} \rightleftharpoons \text{GSSG} + \text{ascorbic acid}$,

where RH_2 = substrate, P = protein residue, G = glutathione residue.

If we consider these suggested processes in the reverse order, reaction (3) is known to proceed. Hopkins & Crook (1938) have shown that ascorbic acid is protected from oxidation in the presence of glutathione which is itself oxidized. Reaction (2) also occurs. Hopkins & Morgan (1938) showed that the thiol groups in succinic dehydrogenase can be oxidized by glutathione and that this inactivates the enzyme. Lens proteins can also reduce glutathione in this way. Reaction (1) has not been demonstrated. The proposed scheme is therefore unproved, but perhaps remains a possible path of lens metabolism.

The results given here suggest that alloxan combines with some of the thiol groups in the lens. Alloxan may have a selective affinity for the thiol groups of the protein, but this seems unlikely; it is more probable that alloxan combines with protein and glutathione in equivalent amounts. If reaction (2) is more rapid than reaction (1), reduced protein (PSH) will be re-oxidized by glutathione (GSSG) as

soon as it is formed. Combination of glutathione with lens protein as suggested by Hermann & Moses (1945) would also explain the maintenance of glutathione concentration at the expense of the protein —SH. A drop in thiol content, or, more accurately, a decrease in the intensity of the nitroprusside reaction has been reported many times, both in clinical and experimental cataract. This change has been attributed to a change in the glutathione concentration, but in view of the results given here it is suggested that this is secondary to a change in the protein of the lens.

This change in reducing sulphur is not, in itself, responsible for the formation of cataract as it occurred in animals that did not show lens changes. All the rabbits that developed cataract had a high blood-sugar level, but out of ten with high blood-sugar levels only five developed cataract. At present no known change either in the chemistry of the lens or in the general metabolism of alloxan-treated rabbits can be shown to lead in all cases to cataract formation. The results reported here showing that an

immediate change occurs in the lens proteins after injection of alloxan make it probable that other early changes also take place. Study of these may be expected to increase our knowledge of cataract formation more than studies on the cataractous lens itself.

SUMMARY

1. Clinical and biochemical changes occurring in the eyes of rabbits during alloxan diabetes are described.

2. An attempt is made to explain the reduction in the amount of protein —SH on the basis of the suggested sulphur-containing oxidation-reduction systems of the lens.

I wish to acknowledge with gratitude my indebtedness to Miss Ida Mann for her advice and encouragement during the early part of this work and for performing the iridectomy operations, and to Dr W. E. Gye for examining the histological specimens. I also wish to thank Dr A. Pirie for her continued guidance and helpful criticism throughout the whole of these investigations.

REFERENCES

- Archibald, R. M. (1945). *J. biol. Chem.* **158**, 347.
 Bailey, C. C., Bailey, O. T. & Leach, R. S. (1944). *New Engl. J. Med.* **230**, 533.
 Bell, E. T. (1948). *Experimental Diabetes Mellitus*. Oxford: Blackwell.
 Bellows, J. G. (1944). *Cataract and Anomalies of the Lens*. London: Kimpton.
 Bellows, J. G. & Rosner, L. (1937). *Amer. J. Ophthalm.* **20**, 1109.
 Bessey, O. A. & King, C. G. (1933). *J. biol. Chem.* **103**, 687.
 Cohen, M., Kamner, M. & Killian, J. A. (1927). *Proc. Soc. exp. Biol., N.Y.*, **25**, 677.
 Dixon, M. (1948). *Biochem. Soc. Symp.* **2**, 46.
 Duffy, E. (1945). *J. Path. Bact.* **57**, 199.
 Dunn, J. S., Sheehan, H. L. & McLetchie, N. G. B. (1943). *Lancet*, **1**, 484.
 Gifford, H. (1932). *Arch. Ophthalm., Chicago*, **7**, 763.
 Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
 Hermann, H. & Moses, S. G. (1945). *J. biol. Chem.* **158**, 33.
 Hopkins, F. G. & Crook, E. M. (1938). *Biochem. J.* **32**, 1356.
 Hopkins, F. G. & Morgan, E. J. (1938). *Biochem. J.* **32**, 611.
 Jacobs, H. R. (1937). *Proc. Soc. exp. Biol., N.Y.*, **37**, 407.
 Kramer, A. & Steiner, H. (1931). *Biochem. J.* **25**, 161.
 Lukens, F. D. W. (1948). *Physiol. Rev.* **28**, 304.
 Perlzweig, W. A. & Delrue, G. (1927). *Biochem. J.* **21**, 1416.
 Pirie, N. W. (1946). Personal communication.
 Reis, W. (1912). *v. Graefes Arch. Ophthalm.* **80**, 588.
 Rosner, L., Farmer, C. J. & Bellows, J. G. (1938). *Arch. Ophthalm., Chicago*, **20**, 417.
 Shoji, Y. (1927). *Ann. Oculist, Paris*, **165**, 344.
 Shoji, Y. (1931). *Arch. Ophthalm., Paris*, **48**, 28.

The Micro-estimation and Origin of Trimethylamine in *Chenopodium vulvaria* L.

By B. T. CROMWELL

Department of Botany, University College, Hull

(Received 21 November 1949)

The presence of trimethylamine in the tissues of *Chenopodium vulvaria* L. was first established by Dessaignes (1852). Further observations on the occurrence of this base in *Ch. vulvaria* were made by Wicke (1862) and Klein & Steiner (1928). Trimethylamine is present in small amounts in the flowers of many plants and occurs with methylamine in the rhizome of *Acorus Calamus* and in the young shoots

of *Mercurialis annua* (Guggenheim, 1940). Challinor (1913) has reported the occurrence of trimethylamine in the tissues of *Rhagodia hastata*, and Kapeller-Adler & Vering (1931) showed that the concentration of trimethylamine may reach the value of 500 mg./100 g. dry weight in the tissues of the red algae *Ceramium rubrum* and *Rhodomela subfusca*. Trimethylamine diffuses from innumerable glands