Factors Affecting the Concentration of Ascorbic Acid in the Crystalline Lens of Cattle and Rabbits

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It has been suggested that ascorbic acid is necessary for the transparency and metabolism of the crystalline lens (see Bellows, 1944). Primarily, this is based on well-authenticated observations of a loss of ascorbic acid from animal and human lenses undergoing spontaneous opacification. In experimentally induced cataract, however, loss of ascorbic acid does not always occur; thus, naphthalene and galactose given orally to rabbits produce cataracts which are accompanied by a decrease in ascorbic acid (Bellows & Rosner, 1937; Rosner, Farmer & Bellows, 1938), and rabbits given alloxan intravenously, or directly irradiated with X-rays, develop cataracts with no significant change in the ascorbic acid of the lens (Waters, 1950; Pirie, Van Heyningen & Boag, 1953).

The cause of the variability of the ascorbic acid level in the cataractous lens is to be found in several factors likely to influence the distribution of ascorbic acid between this tissue and the aqueous humour. The principal factor extrinsic to the lens is the concentration of ascorbic acid in the aqueous humour; this is determined by the rate of secretion of ascorbic acid across the ciliary epithelium (Kinsey, 1947; Langham, 1950; Linnér, 1952; Bárány & Langham, 1953, 1955). There is now little, if any, evidence that synthesis of ascorbic acid occurs in the rabbit lens (Kinsey, 1950; Linnér, 1952; Langham, 1955), but there are two intrinsic factors which could modify the ascorbic acid content, namely permeability and metabolism. That the metabolism of the lens itself might actively inhibit the disappearance of ascorbic acid is suggested by the recent isolation from cattle lenses of the enzyme glutathione reductase which catalyses the reduction of oxidized glutathione by reduced triphosphopyridine nucleotide (Van Heyningen & Pirie, 1953), and the well-known ability of glutathione to reduce dehydroascorbic acid at body pH (Borsook, Davenport, Jeffreys & Warner, 1937).

In the present study the influence of these extrinsic and intrinsic factors on the concentration of ascorbic acid in the lens has been investigated.

METHODS

Experimental procedure

Adult rabbits of both sexes, weighing between 1.8 and 2.5 kg., were used. Eyes were excised immediately after intravenous injection of Nembutal (pentobarbitone sodium B.P.). Aqueous humour was withdrawn into a dry 1 ml. syringe and analysed immediately. All samples of aqueous humour were tested for the presence of protein by the addition of an equal vol. of a solution of 8% (w/v) trichloroacetic acid and were rejected if more than the normal faint clouding was observed. Lenses were excised as described by Langham & Davson (1950).

The ascorbic acid for injections was dissolved in water, partially neutralized with NaOH to a pH of 6.8-7.0 and diluted to an isotonic concentration.

Preparation of lens extracts

Cattle and rabbit lenses were used within 2-3 hr. of death. Whole lenses, usually with their capsules intact, were weighed and finely chopped in a mortar with scissors. They were then ground with a little ballotini and twice their weight of glass-distilled water. This 1 in 3 mush was then transferred to 0.75 in. diameter dialysis tubing (Visking, Chicago) and dialysed for 48-72 hr. against running tap water or large volumes of glass-distilled water or both at 4°C. The insoluble portion was then centrifuged down and discarded.

Analytical methods

Ascorbic acid in the reaction mixtures containing dialysed lens extracts or whole lens was determined by addition of 1 vol. of the reaction mixture to 10 vol. of citrate-phosphate buffer (0.070M citric acid and 0.076M Na_2HPO_4) and titrating with 2:6-dichlorophenolindophenol by the procedure of Langham (1950). In all other studies ascorbic acid (AA), dehydroascorbic acid (DHA) and 2:3-diketogulonic acid (DKG) were analysed by the osazone technique of Roe, Mills, Oesterling & Damron (1948). This method is based on the reaction of DHA and DKG (but not AA) with 2:4-dinitrophenylhydrazine to give an osazone which yields a characteristic red solution in conc. H_2SO_4 . The sample for analysis is divided into three parts: the AA in the first portion is oxidized to DHA; the DHA in the second portion is reduced to AA by H_2S , and the third portion is analysed without prior treatment. From these analyses the concentrations of AA, DHA and DKG may be calculated.

The following modifications of this method were employed. Plasma and lens extracts were made with 2 vol. of a solution containing trichloroacetic acid (8%, w/v) and metaphosphoric acid (1.5%, w/v); AA and DHA added to acid extracts of plasma or lens prepared in this way and left for 1 hr. were recovered quantitatively. (Metaphosphoric acid extracts were found unsuitable owing to the foaming which occurred on gassing with H_2S .) The acid extracts were then adjusted to pH 3.5 by addition of a mixture of 2N-NaOH and 15% (w/v) glycine; reduction of DHA in one sample by H_2S and oxidation of AA in another sample by bromine were carried out at this pH; the rates of reduction of DHA and loss of DKG by H_2S under these conditions are shown in Fig. 1. The adoption of pH 3.5 for the reduction of DHA by H_2S is based on the work of Borsook *et al.* (1937) and Kenyon & Munro (1948), but contrasts with that of Roe *et al.* (1948) who employed pH < 2.0 owing to an observed rapid loss of DKG at pH 3.4; this was not found with the present technique (Fig. 1).

For determination of AA, DHA and DKG in plasma, 1.5 ml. was added to 3.0 ml. of a solution containing 8% (w/v) trichloroacetic acid and 1.5% (w/v) metaphosphoric acid. After centrifuging, 1 ml. of the supernatant was added to each of three tubes (a), (b) and (c) containing 0.1 ml. of 2N-NaOH and 0.25 ml. of 15% (w/v) glycine, and treated as follows: (a) 0.5 ml. of 6% (w/v) thiourea and 1.0 ml. of 9N- H_2SO_4 were added. (b) The mixture was gassed for 45 min. with H_2S , then N_2 was passed through for 5 min., both gases being saturated with water vapour. 0.5 ml. of thiourea solution and 1.0 ml. of 9N- H_2SO_4 were then added. (c) 0.5 ml. of bromine water (1 in 10 dilution of a saturated solution) was added and left for 4 min., and then N_2 was passed through for 10 min. 0.5 ml. of thiourea solution and 1.0 ml. of 9N- H_2SO_4 were then added. 1.0 ml. of 2,4-dinitrophenylhydrazine (2% in 9N- H_2SO_4 solution) was added to all tubes, which were then placed in a bath at 37° for 3 hr. with shaking. The tubes were then removed and cooled in an ether-solid CO_2 mixture for 2 min. and 5.0 ml. of 85% (w/v) H_2SO_4 was run in slowly from a burette. After standing at room temperature for 1 hr., optical transmission of the solution was read against a

reagent blank at 540 m μ . in a cuvette with a 3.0 cm. light path. With a concentration of 1 mg. of AA/100 ml. of plasma an extinction of 0.10 was found. Readings were taken on a Unicam SP. 600 spectrophotometer.

The DHA used was a crystalline preparation containing 1 mol.prop. of methanol. It was stored at 4° over silica gel in a desiccator. Reduction with H_2S gave a yield of 90% of AA. The glutathione was a commercial preparation. Titration with iodine showed it to be 60% pure with respect to SH groups.

RESULTS

Studies on the living rabbit

In a group of ten rabbit lenses having a mean wet weight of 390 ± 10 mg., water content was 61.8 ± 1.1 %

The concentrations of AA in the plasma, aqueous humour and lens of fourteen rabbits on a normal diet are recorded in Table 1. The concentration of AA in the aqueous humour varied from 13 to 40 mg./100 ml., and in this range there was an approximately constant relation between the concentrations in the aqueous humour and the lens. The mean ratio of the concentration of AA in the lens to that in the aqueous humour was 0.79 ± 0.04 (14 observations). The concentration of DHA plus DKG in the lens comprised 15–20% of the total vitamin C. In contrast, in the aqueous humour, DHA plus DKG is less than 2% of the AA concentration (Müller & Buschke, 1934; Langham, 1950, 1955).

In fourteen rabbits equal concentrations of AA were found in the aqueous humour and lenses of

Fig. 1. Reduction of dehydroascorbic acid (●) and 2,3-diketogulonic acid (○) by H_2S at pH 3.5. 200 μ g. of DHA or DKG was added to plasma and the acid extract buffered to pH 3.5 by the addition of NaOH and glycine. The points represent the mean of three experiments.

Table 1. Concentration of ascorbic acid in plasma, aqueous humour and lens of rabbits

The concentration of dehydroascorbic (DHA) plus 2,3-diketogulonic acids (DKG) in the lens is also included. Concentrations of these acids in the plasma and aqueous humour is very low (see Bárány & Langham, 1955). All concentrations are expressed as mg./100 g. of water.

Rabbit	Ascorbic acid			Lens DHA + DKG
	Plasma	Aqueous humour	Lens	
1	0.21	20.0	18.2	1.9
2	0.16	15.8	10.5	2.9
3	0.15	17.3	15.2	4.7
4	0.19	17.7	15.8	2.9
5	0.26	24.2	13.8	4.0
6	0.18	19.9	14.9	1.9
7	0.12	16.6	18.0	2.6
8	0.61	38.8	36.8	7.1
9	0.45	32.4	23.7	4.8
10	0.12	13.3	11.2	2.9
11	0.24	25.2	12.8	2.2
12	0.26	24.9	17.0	3.0
13	0.35	35.0	26.8	3.7
14	0.34	40.0	30.7	6.0
Mean	0.26	24.4	18.9	3.6

Table 2. *Absence of post-mortem changes in the concentration of ascorbic acid in the aqueous humour and lens of the rabbit*

The first aqueous humour (Aq_1) and lens ($lens_1$) were removed at the time of death, and the second aqueous humour (Aq_2) and lens ($lens_2$) after the interval stated. All concentrations are expressed as mg. of ascorbic acid/100 g. of water.

Time after death (min.)	Ascorbic acid				DHA + DKG	
	Aq_1	Aq_2	$lens_1$	$lens_2$	$lens_1$	$lens_2$
60	20.0	20.7	18.3	17.6	1.9	2.7
60	15.8	16.1	10.5	11.0	3.4	2.6
120	19.9	18.5	14.8	14.9	1.9	2.1
120	16.6	17.8	18.0	15.2	2.6	1.9

eyes taken from the same animal. The mean concentrations, expressed as mg./100 g. of water, for the aqueous humour and lens, respectively, were: left eyes, 26.8 ± 2.2 and 19.3 ± 2.0 ; right eyes, 26.9 ± 2.3 and 19.4 ± 2.1 . The average difference in AA concentrations in the aqueous humour of the two eyes of the same animal is significantly less than the value of 4.1 mg./100 ml. observed by Goldmann & Buschke (1935-6) and quoted by Pirie *et al.* (1953) in X-ray studies on the AA content of rabbit lenses.

Changes in the concentration of AA in the aqueous humour and lens after death were studied in four rabbits. The animals were killed and one eye was excised immediately, and the aqueous humour and lens were removed for analysis. The animals were then left, and 1-2 hr. later the second eye was excised. The analyses, recorded in Table 2, show that no significant change in the concentration of AA in the aqueous humour or the lens took place in the 2 hr. period immediately after death.

The effect of a change in the rate of transfer of AA across the ciliary epithelium was studied in three rabbits anaesthetized with urethane. One eye was removed at the start of the experiment. After an initial intravenous injection of 50 mg. of AA, 25 mg. was injected intramuscularly each hour for 5 hr., and the second eye was excised 1 hr. after the last injection. This treatment gives a mean plasma concentration of approximately 10 mg. of AA/100 ml., and is more than sufficient to ensure a maximal rate of transfer of AA into the aqueous humour (Bárány & Langham, 1955; Langham, 1955). The rate of turnover of AA in the aqueous humour is approximately 1-2 %/min. (Becker, Linnér & Bárány, 1951), and a steady state is attained to within a few % in 6 hr. The results tabulated in Table 3 show that there was an increase in the concentration of AA in the aqueous humour but a much smaller proportional rise in the lens.

Studies on the excised lens

Fresh cattle and rabbit lenses with intact capsules inhibited the rate of destruction of AA in ox aqueous humour diluted with Krebs-Ringer

Table 3. *Effect of an increased rate of transfer of ascorbic acid across the blood-aqueous barrier on the concentration of ascorbic acid*

The first aqueous humour (Aq_1) and lens ($lens_1$) were removed initially and the second aqueous humour (Aq_2) and lens ($lens_2$) after 6 hr.

Rabbit	Ascorbic acid (mg./100 g. of water)			
	Aq_1	Aq_2	$lens_1$	$lens_2$
1	23.2	68.7	17.6	23.6
2	18.5	70.0	12.7	22.8
3	26.5	72.5	23.6	31.6

phosphate, pH 7.4, and this activity was enhanced in the presence of added DHA. In ox aqueous humour containing 20 mg. of AA/100 ml. the mean rate of disappearance of AA at 37.5° was 8.8 mg./100 ml./hr. in the absence, and 5.83 mg./100 ml./hr. in the presence, of a lens. This agrees with the findings of Sasaki (1937), who carried out similar experiments using a Ringer-Locke solution. When DHA was added to the medium to give a concentration of approximately 20 mg./100 ml., the rate of loss of AA in the absence and the presence of a rabbit lens was 8.6 ± 0.04 and 2.95 ± 0.02 (5 observations) mg./100 ml./hr., respectively. This agrees with the findings of Huymans & Fischer (1942) but not with those of Kinsey (1950), whose observations were based on reaction times which did not exceed 10 min., and the technique differed in that aqueous humour of cattle eyes was removed and replaced by a solution of phosphate buffer containing DHA. Consequently, the discrepancy could imply that there was a diffusion of reducing substances from the excised lens in the present work. Experiments were therefore made in which DHA was added to media which had previously been kept at 37.5° for 1 hr. in contact with an excised lens. In four experiments a mean decrease of 60 % in the loss of AA from the medium was observed in an experimental period of 15 min. Thus, reducing substances capable of protecting against disappearance of AA are lost by diffusion from the excised lens.

In a final series of experiments a solution of DHA was introduced into the aqueous humour of living rabbits. The aqueous humour was mixed with an isotonic solution of NaCl containing DHA to give a final concentration of approximately 40 mg./100 ml. in the aqueous humour. An identical introduction was made into the other eye of saline solution without DHA. The animals were then left for 30 min. In three animals the mean concentration of AA in the control eyes was 29.5 mg./100 ml. and in the experimental eyes 31.0 mg./100 ml.

The effect of dialysed extracts of cattle and rabbit lenses on the rate of reduction of DHA in the presence of reduced glutathione (GSH) was studied with buffered phosphate media. In control experiments the rate of reduction of DHA (0.0022M) by GSH (0.0123M) in McIlvaine's phosphate-citrate buffer and Sorensen's phosphate buffer was determined. In McIlvaine's buffer reduction rose from zero at pH 3.0 to a very rapid rate at a pH exceeding 6.0, an observation agreeing with that of Borsook *et al.* (1937). Similarly, with Sorensen's buffer the rate of reduction of DHA by GSH increased rapidly at a pH exceeding 7.0. Comparison of the rates of reduction in the two buffers adjusted to a final pH of 7.0 showed that reduction was nearly complete in McIlvaine's buffer while less than 10% was reduced in Sorensen's buffer in 10 min.

The addition of 0.35 ml. of dialysed lens extract (pH 7.2) to a tube containing 0.40 ml. of Sorensen's buffer (0.06M, pH 7.0), 1.55 ml. of water, 0.25 ml. of DHA (1.2 mM) and 0.5 ml. of GSH (1.2 mM) resulted in the formation of AA which was directly proportional to time over 30 min., and a mean production of 33.8 ± 4.15 μ moles of AA/g. of dry tissue/hr. was recorded on eleven extracts. In similar conditions a rate of 41.7 ± 5.0 μ moles of AA/g. of dry tissue/hr. was recorded after keeping the lens extract at 90–95° for 30 min. before the experiment. Ashing of the extract led to a decrease in activity to 6.5 ± 1.0 μ moles of AA/g. of dry tissue/hr. In the absence of added GSH the rate of formation of AA by the lens extract was 15.6 ± 2.5 μ moles of AA/g. of dry tissue/hr.

DISCUSSION

The experimental results show that the concentration of ascorbic acid in the lens of the normal rabbit is modified by at least three variables. First, the rate of transfer of ascorbic acid across the blood-aqueous barrier; secondly, the permeability of the lens to ascorbic acid; thirdly, the rate of reduction of dehydroascorbic acid by substances within the lens. In this discussion it will be assumed that there is no synthesis of ascorbic acid

in the rabbit lens (Kinsey, 1950; Linnér, 1952; Langham, 1955).

A change in the rate of transfer of ascorbic acid across the blood-aqueous barrier has been found to alter substantially the concentration in the lens. In normal rabbits the rate of transfer of ascorbic acid reaches a maximum at a plasma concentration of 2–3 mg./100 ml. and results in a concentration of approximately 70 mg./100 ml. in the aqueous humour (Bárány & Langham, 1953, 1955); at the other extreme, as the plasma concentration approaches zero there is a concentration of 10–14 mg./100 ml. in the aqueous humour. The concentration of ascorbic acid in the lens is approximately 80% of that in the aqueous humour, and in fourteen animals varied from 10 to 36 mg./100 g. of water as the concentration in the aqueous humour varied from 13 to 40 mg./100 ml. (see Table 1). In the presence of a maximal rate of secretion of ascorbic acid into the eye or any decrease in the rate of bulk flow of the aqueous humour, the concentration of ascorbic acid in the lens would exceed 36 mg./100 g. of water.

In view of the complex structure of the tissue and the slow rate of diffusion of radioactive sodium (^{24}Na) and bromine (^{82}Br) into the lens of the adult rabbit (Langham & Davson, 1950) it was to be expected that the time for ascorbic acid to equilibrate between the lens and the aqueous humour would be many hours. This is what has been observed; after increasing the concentration of ascorbic acid in the aqueous humour for 6 hr., the ascorbic acid distribution ratio (concentration in lens/concentration in aqueous humour) was approximately 0.3–0.4, compared with the normal value of 0.79 ± 0.040 . Further, the finding that the concentration of the ascorbic acid transformation products in the lens exceeded that in the aqueous humour suggests that the rate of degradation of ascorbic acid, coupled with a slow overall diffusion of ascorbic acid into the lens, prevents a higher distribution ratio between the two tissues.

It is probable that the reduction of DHA by GSH in the presence of lens tissue is non-enzymic. This view is supported by the observations that the increased rate of reduction of DHA by GSH in the presence of a dialysed extract of the lens was not eliminated by boiling and was due partly to the reduction of DHA by the lens extract itself. A reduction of DHA by the lens extract is consistent with the known presence of protein-containing SH groups in the lens (Bellows, 1944; Pirie *et al.* 1953). An alternative explanation that GSH was not completely removed is unlikely, as increasing the period of dialysis did not eliminate the reaction.

Normally, there is in rabbit lenses an average concentration of 320 mg. of GSH/100 mg., and in

the development of cataract there is evidence that this level decreases before any visible change takes place in the lens (Pirie *et al.* 1953). Concurrent with this decrease in GSH a fall in the activity of the enzyme glutathione reductase has been observed (Pirie *et al.* 1953). Consequently, in the cataractous lens the stability of ascorbic acid would tend to decrease and the proportion of DHA plus DKG to increase. Similarly, the resultant effect of a cataractous agent on the concentration of ascorbic acid in the lens will be principally determined by its action on the transfer of ascorbic acid across the blood-aqueous barrier and on the permeability of the lens to ascorbic acid; this is illustrated in reported studies on experimental cataract. In naphthalene-induced cataract Müller & Buschke (1934) found that the concentration of ascorbic acid in the aqueous humour and the lens approached zero but there was an absolute increase in the concentration of DHA in the aqueous humour. This suggests that the decreased level of ascorbic acid in the lens is due to the action of naphthalene in impeding the accumulation of ascorbic acid in the aqueous humour. In contrast, the investigations of Waters (1950) on alloxan cataract and of Pirie *et al.* (1953) on X-ray cataract indicate that the accumulation of ascorbic acid in the aqueous humour is unimpaired and that no significant decrease takes place in the concentration of ascorbic acid in the lens, even though a fall in the glutathione-reductase activity was observed after X-ray irradiation.

SUMMARY

1. The distribution of ascorbic acid between the plasma, aqueous humour and lens of normal rabbits has been determined. The concentration of ascorbic acid in the lens has been found to be approximately 80 % of that in the aqueous humour when the latter varied from 13 to 40 mg./100 ml. The concentration of dehydroascorbic plus 2:3-diketogulonic acids in the lens was 15–20 % of the total vitamin C.

2. Ascorbic acid concentrations in the lens and aqueous humour of the two eyes of the same rabbit did not differ significantly. Average concentrations of ascorbic acid in the aqueous humour were 24.4 mg./100 g. of water and in the lenses 18.9 mg./100 g. of water. No change in the concentration of ascorbic acid in the eye was observed in the period 2 hr. after death.

3. The effect of an increased rate of transfer of ascorbic acid across the blood-aqueous barrier on the concentration of ascorbic acid in the lens has been determined. The results suggest that both the capsule and lens fibres are permeable to ascorbic acid.

4. The reduction of dehydroascorbic acid by whole rabbit and cattle lenses and lens extracts has been studied. Whole lenses were found to inhibit ascorbic acid loss in ox aqueous humour and Krebs-Ringer phosphate, an action which is particularly marked in the presence of added dehydroascorbic acid. In dialysed cattle- and rabbit-lens extracts no evidence was found for an enzyme which activates the reduction of dehydroascorbic acid by glutathione.

5. The importance of the permeability of the lens, the rate of transfer of ascorbic acid across the blood-aqueous barrier and of lenticular metabolism on the concentration of ascorbic acid in this tissue is discussed. It is concluded that the concentration of ascorbic acid in the lens is determined principally by the rate of transfer of ascorbic acid into the eye and the permeability of the lens to ascorbic acid.

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