CCIII. CATARACT AND ASCORBIC ACID IN THE GUINEA-PIG EYE.

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The occurrence of vitamin C in the aqueous humour and lens in relatively large amounts, first noted by Birch & Dann [1933; 1934] in the case of the ox and sheep, has attracted much attention by workers interested in the genesis of cataract. Thus Müller *et al.* [1933; 1934; 1935] found that the vitamin content of both the aqueous humour and lens of cattle and rabbits decreased with age and on the development of cataract. The latter workers as well as Fischer [1934] not only associated the vitamin with the respiratory activity of the lens but suggested that it was most probably synthesized in that organ, at least of young animals. However, although Fischer found that derangements of the respiratory function resulted in the formation of cataracts, he was unable to state whether the disappearance of the vitamin preceded or followed the development of the cataract. The view that the lens could synthesize the vitamin was questioned by Van Eekelen *et al.* [1934] who inclined to the belief that it was accumulated there by a selective secretory process.

Similar variations in the vitamin content of the eyes of other animals and man due to the same causes have been observed by Euler and co-workers [1933; 1934].

The above results were obtained chiefly by titration of extracts with indophenol but the presence of the vitamin in the humours and lens of the normal eyes of the ox was demonstrated biologically by Birch & Dann [1934], Euler & Malmberg [1934] and more recently by Müller & Demole [1935].

It is now well established that the guinea-pig quickly loses its store of vitamin C when maintained on a scorbutic diet. The organs of this animal which are known selectively to absorb it have been found to lose their individual stores simultaneously and to become depleted in about the same time. Since the amount of vitamin available for distribution in the body of the guinea-pig can be readily controlled, it was of interest to determine whether the eye behaved similarly to the other organs in this respect in the hope of contributing to the solution of the above problem.

The problem was considered from two aspects. One the fate of the ascorbic acid in the eye of the guinea-pig during the process of depletion of vitamin C and the other its occurrence in the eyes of the depleted animals following the administration of the vitamin.

Owing to the small quantities of material available, biological methods of establishing the presence or of determining the amounts of vitamin C in the lens of the guinea-pig were not applicable to the present work. The indophenol titration method whilst simple tends to give untrustworthy results when used with extracts of tissues, such as the lens, which contain sulphydryl compounds. The spectrophotometric method was therefore employed in conjunction with the titrimetric method in order to obtain greater specificity.

Trichloroacetic acid, which was used by Van Eekelen *et al.* [1934] and Plaut *et al.* [1935] as a protein-precipitating agent in their work on the humours and cerebrospinal fluid, is not suitable for the preparation of extracts in these determinations because of the opacity of its solutions in the region below about 260 $m\mu$ where ascorbic acid shows selective absorption. Freshly redistilled absolute alcohol was therefore employed not only on account of its transparency but also because of the stability which it confers upon ascorbic acid.

Experimental.

The spectrograph, light source etc., which were employed in this work have already been described in another paper from this laboratory [Kellie & Zilva, 1936].

A value of 10,000 was obtained for the molecular extinction coefficient of pure ascorbic acid in $N/100$ HCl in 90 % alcohol. In this solution the head of the absorption band occurred at 245 $m\mu$. All determinations were made on acidified solutions and the above value was used in estimating the amounts of ascorbic acid.

Titrimetric and spectrophotometric determinations of ascorbic acid.

The humours and lenses of the horse, ox, sheep and pig, as well as those of the guinea-pig, were examined.

Determinations in the aqueous humour. The humours were collected in an injection syringe by puncture of the cornea near to the centre of the pupil, the needle being inserted obliquely so as to avoid injury to the iris and lens. They seldom needed to be clarified but when necessary they were filtered or centrifuged.

For the titrimetric determination the humour was acidified with one-fifth of its volume of glacial acetic acid and titrated directly with indophenol. The latter was standardized so that 10 ml. of the indicator solution were equivalent to 1·0 mg. of ascorbic acid.

In the spectrographic determination a dilution of 1 in 10 generally sufficed to give the required concentration of 0·5–2·5 mg. of ascorbic acid per 100 ml. Two equal volumes of humour, usually 1·0 ml., were necessary. One was immediately acidified with 0·2N HCl and diluted with fresh glass-distilled water to give the desired concentration of humour in 0·02N HCl. The ascorbic acid in the second sample was oxidized by diluting it with water to about 80 % of its final volume and adding sufficient $CuSO_4$ solution to give ultimately a concentration of 2 mg. of Cu per litre of solution. At the reaction of these solutions, which was invariably neutral, the oxidation was complete in an hour. It was then acidified and the acid concentration adjusted as in the previous case. The absorption spectrum of each solution was measured by comparing the first with an equal depth of 0·02N HCl and the second with a similar solution containing 2 mg. of Cu per litre. This procedure allows the absorption due to the reagents to be balanced during the determinations and thus to be virtually eliminated from the photographs. The difference between the curves for the two solutions gives a measure of the absorption due to ascorbic acid.

Determinations in the vitreous humour. The vitreous humour and lens were removed together by pressing in the cornea after slitting the sclerotic. They could then be easily separated from one another. The humour was freed from the fine fibrous mesh and other insoluble material by filtering it with gentle suction through a small tight pad of glass wool. Any excess protein in the filtrate could

be readily precipitated by adding one drop of glacial acetic acid per ml. of humour.

The spectrophotometric and titrimetric determinations were carried out in the same manner as in the case of the aqueous humour.

Determinations in the lens. The lenses were freed from adhering humour with filter-paper. Titrations were carried out on trichloroacetic acid extracts, which were prepared by thoroughly grinding the lenses in the first place with powdered glass and an equal weight of water and then with five volumes of 5 % trichloroacetic acid.

Alcoholic extracts were used for the spectrophotometric determinations and in calculations the lenses were considered to contain 60 % of water. The lenses were ground with powdered glass and 40 % of their weight of water. The well ground mass, whilst vigorously stirred, was slowly diluted with freshly redistilled absolute alcohol until the concentration of the latter became 90 %. The protein, which commenced to separate when the concentration of alcohol rose above 50 %, was removed on the centrifuge. Other material which reduced the transparency of the solutions in the essential ultra-violet region was precipitated by the addition of 2 % of a 1·5 % solution of $CdCl_2$ in 98 % alcohol. The final extracts were invariably neutral in reaction.

Observations made at the essential stages indicated that no appreciable losses of ascorbic acid occurred during the extraction. Table I shows that ascorbic acid in a solution of the lens material was quite stable and almost completely protected from oxidation by relatively large amounts of Cu at *p*H 7·6. There

Table I.

Time in hours	ml. *N*/1000 indophenol reduced by 1·0 ml. solution containing 13 mg. ascorbic acid per 10 ml. of 2 % lens material. Solution kept at *p*H 7·6	
	Solution alone	Solution containing 0·1 mg. Cu per 10 ml.
0	15·5	15·5
3	14·0	14·0
7	13·0	13·0
20	12·0	10·0

was therefore little risk of it becoming oxidized whilst the lenses were being ground. The coincidence of the absorption curves in Fig. 1 demonstrates that no significant losses occurred during the subsequent manipulations. One of the curves refers to an extract of the lens to which ascorbic acid was added before precipitation with alcohol and the other to an extract of the same material to which an equal quantity of ascorbic acid was added after the precipitation. Such agreement between the two curves is only possible when no loss of ascorbic acid is involved in the precipitation.

In the spectrographic determination, the oxidation was carried out by adding sufficient Cu to give a concentration of 4 mg. per litre. Although ascorbic acid is normally quite stable in alcoholic solution it oxidizes in alcohol at a much quicker rate than in water on the addition of Cu, presumably owing to the much larger amounts of oxygen dissolved by the former. The freshly prepared and oxidized extracts were acidified with 25 % of their volume of 0·2*N* HCl. When each extract was balanced against its corresponding blank solution, as in the case of the humours, more satisfactory results were obtained with the extracts

of the lens of the horse, ox, sheep and pig than with those of the guinea-pig. In contrast to the others it was found that in the guinea-pig extracts the absorption due to ascorbic acid was small compared with the total absorption. This difficulty was overcome by comparing the oxidized and untreated extracts together in the photometer, which virtually eliminates the absorption due to the material other than ascorbic acid in the extracts. It was then necessary to reduce the amount of Cu to 1/10th of that previously employed and to determine its absorption also, for although the latter is very small in dilutions of this magnitude it cannot be ignored when the absorption to be measured is not great.

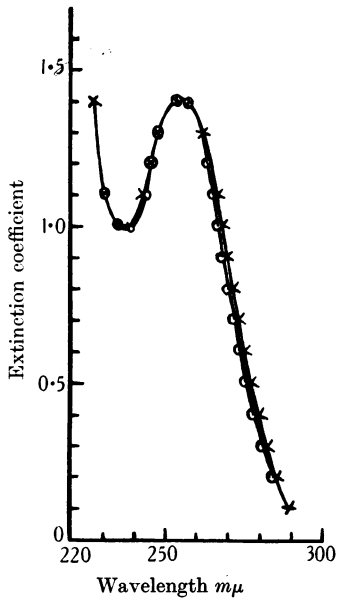

Fig. 1.

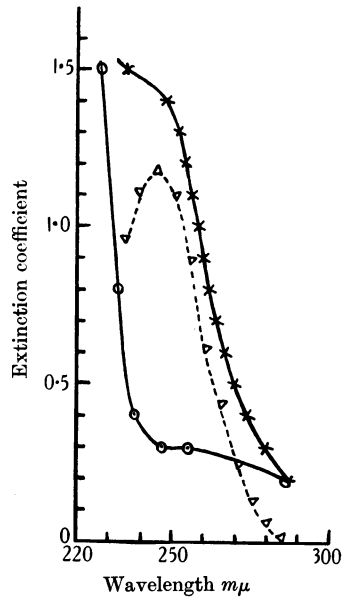

Fig. 2.

Fig. 1. Guinea-pig lens extract containing 12·5 mg. added ascorbic acid per 100 g. of lens. Extract equivalent to 4 g. lens per 100 ml. ×—× Ascorbic acid added before precipitation. o—o Ascorbic acid added after precipitation.

Fig. 2. Ox aqueous humour, dilution 1/10. Ascorbic acid = 21 mg./100 ml. (titration showed 21·6 mg./100 ml.). ×—× Humour. o—o Humour after oxidation. Δ—Δ Ascorbic acid.

Results.

Good agreement between the results of the spectrophotometric and indophenol titration methods was found to exist only in the case of the humours. The titration values obtained for the lenses were invariably higher than those of the spectrographic method (Table II). The absorption curves in Figs. 2, 3 and 4 are typical and illustrate this fact. In this connexion it must be mentioned that the biological tests of Müller & Demole [1935] showed that whereas the ascorbic acid content of the humours of the ox eye was truly indicated by the indophenol method, that of the lens only accounted for about a half of its indophenol-reducing capacity. It may therefore be concluded that whilst the titrimetric method could not be applied in the determination of ascorbic acid in the lens, the

Table II.

Animal	Tissue	Ascorbic acid in mg. per 100 ml. or per 100 g. Indophenol titration	Spectrophotometric determination
Horse	Aqueous humour	16·6	18·0
	Vitreous humour	9·0	12·0
Ox	Aqueous humour	21·6	21·0
	Vitreous humour	15·0	10·0
Sheep	,, ,,	13·5	10·0
Pig	,, ,,	5·0	5·5
Guinea-pig	,, ,,	21·3	20·0
,,	,, ,,	18·5	19·0
Horse	Lens	37·0	26·0
Ox	,,	32·6	22·5
Sheep	,,	21·0	17·5
Pig	,,	26·0	13·5

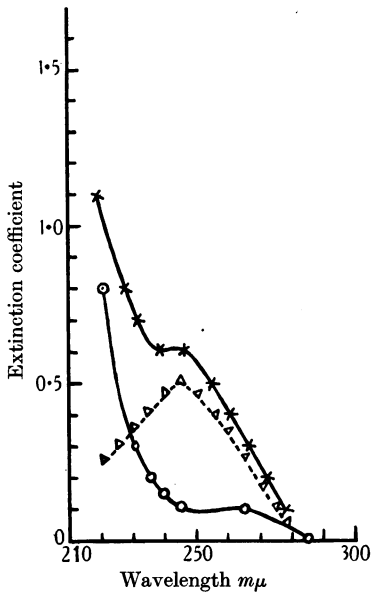

Fig. 3.

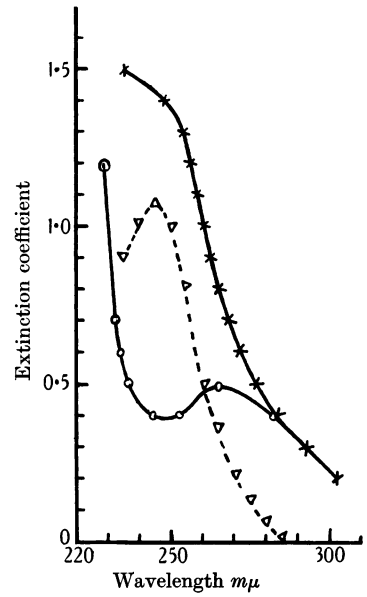

Fig. 4.

Fig. 3. Ox lens extract, equivalent to 4·4 g. lens per 100 ml. Ascorbic acid = 22·5 mg./100 g. lens (titration showed 32·6 mg./100 g. lens). ×—× Extract. o—o Extract after oxidation. Δ—Δ Ascorbic acid.

Fig. 4. Guinea-pig vitreous humour, dilution 1/10. Ascorbic acid = 19 mg./100 ml. (titration showed 18·5 mg./100 ml.). ×—× Humour. o—o Humour after oxidation. Δ—Δ Ascorbic acid.

spectrophotometric method showed adequate specificity for this purpose. Consequently the titration method sufficed in the case of the humours but both methods were employed for comparison in the examination of the lens.

The fate of ascorbic acid in the humours and lens of the guinea-pig during depletion of vitamin C.

The guinea-pigs employed in this work were taken from a stock which had subsisted for a long period on a normal mixed diet of oats, bran and cabbage *ad lib.* They were divided into three groups each consisting of 12 animals of

similar age and weight. The eyes of the first group were used as controls and were examined immediately. The second and third groups were maintained on scorbutic diets for 5 and 9 days respectively. All the animals were killed by stunning and bleeding and their eyes removed and examined without delay. No macroscopic abnormalities were observed in any of the eyes.

Table III.

Group	Average weight g.	Diet	Duration of experiment days	Titrimetric determination ascorbic acid in humours mg. per 100 ml.		Ascorbic acid in lens mg. per 100 g.	
				Aqueous	Vitreous	Titrimetric determination	Spectrophotometric determination
1	390	Normal	—	13·0	12·0	8·0	4·0
2	398	Scorbutic	5	0·5	2·5	4·7	1·5
3	404	„	9	0·5 (?)	0·7	4·0	0·0

It will be seen from Table III that the concentrations of ascorbic acid in the two humours of the animals of the control group were about equal but greater than that in the lens. In the latter the concentration of the vitamin determined spectrographically amounted to only half of that indicated by the titration. In group 2 the concentration in the lens as determined by both methods had fallen but the titration value remained much higher than that of the spectrographic method. The value found by the spectrograph in this case is only approximate owing to the low level to which the concentration had fallen. The loss of the vitamin from the humours of the animals of this group was also very marked; it was almost complete in the aqueous humour but an appreciable amount remained in the vitreous humour. No ascorbic acid was detectable by the spectrographic method in the lenses of the last group of animals in spite of the fact that the indophenol titration method indicated a reducing capacity equal to half that of the control group. This residual reducing capacity happens to be equal to the difference between the amounts of ascorbic acid determined by titration and by the spectrograph in the case of the animals of the control group which strongly indicates that the fall in the reducing capacity during the period of depletion is due to ascorbic acid alone.[1] Both the humours of the last group were practically devoid of ascorbic acid.

The above results show that the humours and lens resemble the other tissues in their inability to conserve their stores of ascorbic acid when the animals are maintained on vitamin C-deficient diets. It is concluded from the rate at which it occurs that the loss of the vitamin from the eye runs parallel with that of the body generally. This conclusion is contrary to the view expressed by Ray *et al.* [1935] that the lens of the scorbutic guinea-pig holds tenaciously to a part of its vitamin C content. They arrived at this conclusion by the application of the titrimetric method only. In view of the above results the residual indophenol-reducing capacity of the lens observed by them, which in any case seems rather high, was evidently not due to ascorbic acid.

The occurrence of ascorbic acid in the eyes of vitamin C-depleted guinea-pigs following its administration.

The distribution of the vitamin in the avascular parts of the eye can be approximately followed from the ascorbic acid concentrations in the humours which nourish them since it has been shown that the lens and humours are

[1] Only approximate relations between the corresponding figures can be expected since the changes occurring in the eyes cannot be examined in the same animals at each stage.

analogous in their behaviour to one another and to the other tissues which selectively absorb it. In order therefore to simplify the experimental procedure, titrations of the vitreous humour only were carried out in the following experiments. Seven groups of two animals each, similar to those previously employed were used in this work. They were all maintained on a scorbutic basal diet. After the preliminary period of depletion on this diet the animals of three of the groups received ascorbic acid *per os* and the remainder by intramuscular injection. In all cases the animals were killed in the same manner as those in the previous experiment 24 hours after being given their last dose. The eyes of all the animals, none of which showed any clinical signs of scurvy, appeared to be quite normal at the end of the experimental periods. I am indebted to Dr S. S. Zilva for having kindly placed at my disposal for this work the eyes of animals which were being used by him in another investigation.

Table IV.

Group	Average weight g. Beginning of exp.	Average weight g. End of exp.	Preliminary period on scorbutic diet days	Daily dose of ascorbic acid mg.	Number of doses	Method of administration	Ascorbic acid in vitreous humour mg. per 100 ml.
1	283	396	10	0·5	20		0·5
2	270	365	6	15·0	14	*Per os*	16·0
3	293	320	5	25·0	3		8·0
4	265	298	5	8·0	1		5·0
5	275	290	5	5·0	1	Intramuscular injection	3·0
6	263	279	5	8·0	1		7·0
7	266	280	5	5·0	1		4·0

Table IV shows that in the animals in group 1, which were given a prophylactic dose of 0·5 mg. of ascorbic acid for 20 days after a preliminary period of depletion lasting for 10 days, only minimum amounts of ascorbic acid had been accumulated in the vitreous humour. The concentration of the vitamin in the humour of the second group of animals was about normal at the end of 14 days. They had received thirty times the prophylactic dose daily after a much shorter period of depletion. The animals of the third group were depleted for 5 days and then given 25 mg. of the vitamin on each of the following 3 days. In their case the concentration in the humours rose to only half of that of the previous group. The remaining animals, which received a single dose by injection on the 6th day, were arranged in two pairs of groups so that the second pair were duplicates of the first. One group in each pair was given 8 mg. and the other 5 mg. As will be seen from Table IV, the concentration in the humour appeared to rise more rapidly on injection than after oral administration. These latter results are in consonance with those found by Zilva [1936] for other tissues of the same animals.

The manner in which the concentration of ascorbic acid in the vitreous humour of vitamin C-depleted animals varies with the method, size and frequency of administration of the dose is strictly comparable with that which has been observed to occur in other organs and tissues under the same conditions.

The loss of indophenol-reducing capacity and characteristic absorption in the lens and humours is unlikely to be due to the conversion of the vitamin into dehydroascorbic acid for the following reasons. It was seen that the lens material, like many other tissues, exercised a marked stabilizing action on ascorbic acid in solution, protecting it from oxidation. Further, Goldmann & Buschke [1935] state that dehydroascorbic acid is reduced by the lens when it is introduced into the aqueous humour. None of the tissues so far examined has

been found capable of dehydrogenating ascorbic acid *in vitro* [Kellie & Zilva, 1935]. Finally, when dehydroascorbic acid is administered the body was shown to reduce it [Johnson & Zilva, 1934].

SUMMARY.

Good agreement was found between the indophenol titration and spectrophotometric methods of determining the concentration of ascorbic acid in the humours of the eyes of the horse, ox, sheep, pig and guinea-pig. In the case of the lenses, however, indophenol titration invariably gave results which were higher than those of the spectrographic method.

In guinea-pigs on scorbutic diets, the indophenol-reducing capacity of the humours fell rapidly and was almost nil after 9 days. At the end of the same period there remained in the lens some indophenol-reducing capacity which was shown by the spectrograph not to be due to ascorbic acid.

The level of concentration of ascorbic acid in the humours of vitamin C-depleted animals can be raised by the injection or oral administration of ascorbic acid.

The rates of disappearance of the vitamin from the lens and humours during depletion and of its reappearance in the humours on readministration run parallel therefore with those of the other tissues of the guinea-pig.

No cataracts were observed in any of the eyes of the apparently normal animals (those which were maintained for short periods on scorbutic diets) whose humours and lenses were found to be devoid of vitamin C, or even as late as the premortal stage in groups of guinea-pigs suffering from well declared scurvy.

The improbability of the presence of dehydroascorbic acid in the lens and humours is stressed.

It is concluded that the deprivation of guinea-pigs of vitamin C has no direct bearing on the aetiology of cataract in these animals.

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