'amino'-N represented 85·4 % of the total N, and although about 0·3 % of the total N yet remained to be liberated as 'amino'-N, some 1·4 % had already appeared as NH_3 (in addition to amide-N) from degradation of amino-acids.

4. Tests indicated that volatile amines (substituted ammonia) could have been present only in traces in the hydrolysate, and the conclusion has been drawn that when an amino-acid is decarboxylated under the above conditions, the amino group also must usually be involved or the amine first formed must be deaminated rapidly.

5. Organic acids arising from decomposition of the impurity (believed to be a polysaccharide or polyuronide, like mucilage) and by deamination of amino-acids, were found to be more readily removed by prolonged extraction of the hydrolysate with ether, than by repeated evaporation with water at the boiling-point under low pressure.

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Ascorbic Acid Content of Cornea

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In several animal species the aqueous humour contains 20–30 times as much ascorbic acid as the blood plasma. This was first shown by Müller (1933) and the average values collected by Bellows (1944) are: cattle 20, rat 15, rabbit 25, and guinea-pig 13 mg./100 ml. This high concentration in a body fluid is exceptional for, in general, ascorbic acid is more concentrated in the body cells than it is in the fluids. For example, Ralli & Sherry (1941) found that human white blood cells contain 25–38 mg. ascorbic acid/100 g., while average values for human plasma are below 1·0 mg./100 ml.

Other parts of the eye also contain high concentrations of ascorbic acid. The retina (Sullmann & Schmidt, 1942), the vitreous humour (Johnson, 1936) and the ciliary processes (Friedenwald, Buschke & Michel, 1943) in cattle, rabbits and guinea-pigs have about 15–20 mg./100 g.: the concentration in the lens may be higher or lower than it is in the aqueous humour according to the species of animal studied (Bellows, 1944). Friedenwald *et al.* (1943) consider that ascorbic acid is secreted into the aqueous humour by the ciliary body and that this is the source of the ascorbic acid in the lens. The aqueous

humour bathes the internal surface of the cornea as well as the lens surfaces and it seemed of interest to determine the concentration of ascorbic acid in this tissue. In the first place the normal values may give us information about the permeability of the corneal endothelium and epithelium to a weak acid which is a normal constituent of the aqueous humour. In the second place it is of general interest to know the normal concentration of ascorbic acid in the cornea and what change takes place after injury as Wolbach (1933) has shown that ascorbic acid is necessary for the formation of collagen which is the main constituent of the corneal stroma.

The ascorbic acid content of the cornea in cattle and rabbits has therefore been investigated as well as the change in its concentration after a chemical injury. The permeability to ascorbic acid of the corneal endothelium and epithelium has been briefly studied in an attempt to determine the source of the ascorbic acid in the cornea and how it is related to that in the aqueous humour.

METHODS

Rabbit cornea. The two eyes were excised immediately after death and the corneas quickly cut out, blotted free of aqueous humour, weighed and finely minced with scissors. The minced tissue was transferred to a centrifuge tube and 0·5 ml. of 30% metaphosphoric acid was added. The tissue was ground up in this with a glass rod, 1·5 ml. water was added and the tube was then centrifuged. The supernatant liquid was titrated from a 1·0 ml. burette with 0·05 or 0·1 ml. 2:6-dichlorophenol-indophenol which was freshly prepared each day. The concentration of the dye was such that 0·1 ml. dye was equivalent to 0·01 mg. ascorbic acid. In four experiments the corneal epithelium was separated from the stroma by scratching it off with a sharp knife and the ascorbic acid content of the epithelium and stroma was separately estimated.

Ox cornea. The ascorbic acid content of whole single corneas and of separated epithelium and stroma was estimated. The tissue was first chopped, weighed and then ground with sand in a cooled mortar. Metaphosphoric acid (1 ml. 30%) and 3 ml. water were then added and, after centrifuging, the titration was carried out in the usual way.

Aqueous humour. The aqueous humour was taken with a hypodermic syringe or small capillary pipette from the rabbits' eyes before and from the ox eyes after excision. The volume was measured and an equal volume of 30% metaphosphoric acid was added. The total volume was brought to 1 ml. with water and after centrifugation was titrated with 0·05 or 0·1 ml. dye. The volume of aqueous humour obtainable from a rabbit's eye was approximately 0·2 ml.

The metaphosphoric acid extracts from the corneas were usually faintly opalescent. To check whether this opalescent material had any effect on the titration of ascorbic acid, fresh ox corneas were chopped, weighed and then dialyzed against a large volume of distilled water at 0° for 48 hr. before being extracted with acid. The extracts from these dialyzed corneas although opalescent contained no substance which titrated with the dye.

RESULTS

The average concentration of ascorbic acid found in rabbit cornea was 55 mg./100 g. of tissue with a range from 33 to 81 mg./100 g. Cattle cornea had an average concentration of 31 mg./100 g. of tissue with a range of 26 to 36 mg./100 g. (Table 1).

Table 1. *Ascorbic acid content of cornea*

No.	Ascorbic acid (mg./100 g.)	Wt. of pair of corneas (g.)	No.	Ascorbic acid (mg./100 g.)	Wt. of a single cornea (g.)
	(*a*) Rabbit cornea			(*b*) Cattle	
1	53	0·125	1	26	0·47
2	33	0·165	2	32	0·51
3	45	0·165	3	36	0·55
4	54	0·140	4	32	0·51
5	64	0·165	5	31	0·52
6	47	0·160	Average	31	0·51
7	68	0·135			
8	48	0·125			
9	73	0·135			
10	60	0·130			
11	81	0·130			
12	60	0·115			
13	41	0·150			
14	40	0·130			
Average	55	0·140			

The concentration in the corneal epithelium is much higher than it is in the stroma (Table 2). Ox corneal epithelium contains about half of the total ascorbic acid present in the cornea although it is

Table 2. *Ascorbic acid content of corneal epithelium and stroma*

No.	Ascorbic acid (mg./100 g.) in Epithelium	Stroma	Aqueous humour
	(*a*) Rabbit		
1	93	24	29
2	150	36	40
3	118	23	34
4	111	16	23
	(*b*) Cattle		
1	94	17	—
2	90	17	—
3	59	16	—
4	47	17	—

only about a sixth of the total weight. The concentration in the corneal epithelium of the rabbit seems to be even higher, but owing to the small weight of tissue obtainable (*c.* 15 mg. from two eyes) the figures are probably not as reliable as those for the ox. In both animals the concentration in the stroma is much the same as the concentration in the aqueous humour. The high concentration in the epithelium may be a reflexion of its high cell content for ascorbic acid is concentrated in the body cells. The concentration of riboflavin is also higher in ox corneal

epithelium than it is in the stroma but, in this case, the concentration is very low both in the corneal stroma and in the aqueous humour (Philpot & Pirie, 1943).

These results show that the concentration of ascorbic acid in the cornea is considerable but do not enable us to decide whether its source is the aqueous humour or whether the corneal epithelium, which has the highest concentration of any part of the eye, is the source of that in the corneal stroma and possibly also of that in the aqueous humour. In order to get evidence on this point the permeability of the corneal endothelium to ascorbic acid was tested and the change in the concentration of ascorbic acid was determined in the cornea and aqueous humour after injury to the cornea.

Penetration of corneal endothelium by ascorbic acid

Experiments were done to see whether the passage of ascorbic acid through the corneal endothelium from the aqueous humour could be demonstrated. Bellows (1936) found that the concentration in the aqueous humour was raised 1 hr. after 100 mg. of ascorbic acid had been put in the conjunctival sac of a rabbit's eye, which shows that the cornea is permeable in at least one direction. Neutralized ascorbic acid, 2–4 mg., was injected into the anterior chamber of ox eyes brought from the slaughter-house immediately after the death of the animal. The eyes were left in a moist vessel on the bench for 10 min. and the corneas were then excised, washed, blotted, weighed and then extracted as usual for the estimation of ascorbic acid. Table 3 shows that there was a small rise in the ascorbic acid content of the corneas.

Table 3. *Penetration of cattle cornea by ascorbic acid*

No.	Ascorbic acid injected mg.	Ascorbic acid injected ml.	Ascorbic acid found (mg./100 g.)
1	2	0·1	38
2	2	0·1	51
3	4	0·2	57
4	4	0·2	60
5	4	0·2	38
6	8	0·2	61
Average value and range for normal cornea			31 (26–36)

A further attempt to test the permeability of the endothelium was made by determining whether removal of aqueous humour lowered the concentration of ascorbic acid in the cornea. The aqueous humour was removed from both eyes of the rabbit with a sterile capillary pipette after cocainizing the eye and the ascorbic acid concentration was estimated. The aqueous humour was again removed after 60 min. and again after 150 min. and the ascorbic acid content of these reformed aqueous humours was estimated. After 60 min. the concentration had fallen to one-half and it remained at this level. 30 min. later the rabbit was killed and the ascorbic acid of the corneal stroma and epithelium was estimated (Table 4).

Table 4. *Change in concentration of ascorbic acid after removal of aqueous humour. Left eye*

Time (min.)	Volume of aqueous humour (ml.)	Concentration of ascorbic acid: Aqueous humour (mg./100 ml.)	Corneal stroma (mg./100 g.)	Corneal epithelium (mg./100 g.)
0	0·22	23	—	—
60	0·18	10	—	—
150	0·16	11	—	—
180	—	—	11	89
Average and range of four normals		—	25 (36–16)	118 (150–93)

The fall in ascorbic acid in the cornea is not sufficient to account for the replacement of the aqueous humour ascorbic acid, and the results show that, under the conditions of the experiment, there is very little diffusion of ascorbic acid from corneal epithelium to aqueous humour. The fall in concentration in the corneal stroma may be significant and indicate permeability of the endothelium. The drawbacks of the experiment are that the effect of cocaine on the permeability of the corneal cells is unknown and the reformed aqueous humour contains considerable protein and cannot be considered as a normal bathing fluid for the corneal endothelium.

An experiment to find whether the corneal epithelium was permeable to ascorbic acid in the outward direction showed that little, if any, diffused out of the epithelium in 2 hr. at 20°. Washed, excised ox corneas were suspended in 3·0 ml. of phosphate Ringer solution, pH 7·4, so that the epithelium was completely bathed by it. The whole cornea was enclosed in a moist chamber. After various times the ascorbic acid of the cornea, epithelium and bathing fluid was determined. There was no change after 30, 60 or 120 min., all values being within the normal range. No ascorbic acid could be detected by titration of the bathing fluid.

Effect of injury

A series of experiments was carried out to see what change took place in the ascorbic acid concentration of the cornea after injuries which cause oedema of the substantia propria and destruction of the corneal corpuscles. The oedema fluid that enters the injured cornea can come from the aqueous humour, the capillaries at the limbus, or from the tears. Determination of the ascorbic acid concentration of the injured cornea might give a clue to

the source of the oedema fluid. The corneas of rabbits were injured with $\beta\beta'$-dichlorodiethyl sulphide. Pullinger & Mann (1943) have shown that this substance causes oedema of the rabbit's cornea which may be maximal after the epithelium has healed by sliding of the peripheral epithelial cells over the denuded area. The technique was exactly that of Mann & Pullinger (1942). Development of the oedema was watched with a slit lamp and could finally be judged from the weight of the excised cornea. The ascorbic acid content of the cornea and aqueous humour was estimated in the usual way. In all cases the aqueous humour contained large amounts of protein precipitable by metaphosphoric acid.

Table 5 shows that the concentration of ascorbic acid has fallen until it approximately equals that of the aqueous humour. The weight of the cornea may increase 2–3 times and, if the oedema fluid were aqueous humour one would not expect such a fall in ascorbic acid concentration, on the assumption that the capacity of the injured cornea to hold ascorbic acid remains normal. This assumption, however, is probably unjustified, for Pullinger & Mann (1943) showed that such injury destroyed the corneal corpuscles, the only cells of the corneal stroma. Four days after injury, the time at which most of the estimations were done, the epithelium has healed by sliding but no regeneration of corneal cells will have taken place. We cannot conclude, therefore, that the large fall in the concentration of ascorbic acid in such injured corneas means that the oedema fluid does not come from the aqueous humour.

Table 5. *Concentration of ascorbic acid in cornea and aqueous humour after injury*

		Ascorbic acid concentration		
No.	Time after injury	Aqueous humour (mg./100 ml.)	Corneas (mg./100 g.)	Wt. (g.)
1	7 hr.	37	44	0·175
2	1 day	23	24	0·275
3	2½ days	13*	8*	0·30
4	4 days	25	16	0·27
5	4 days	26	21	0·30
6	4 days	18	22	0·44
	Normal average	26	55	0·14

* This rabbit developed iridocyclytis.

A further opportunity to test the effect of injury on the corneal ascorbic acid came when one rabbit in the colony developed bilateral cataract due to a previous intraocular injection into the vitreous humour. The ascorbic acid of the lens and aqueous humour is known to be lowered in some types of cataract and the eyes of this animal were used to determine what the concentration of ascorbic acid might be in the normal cornea of an abnormal eye. Neither the lens nor the aqueous humour contained titratable amounts of ascorbic acid. The corneas contained approximately 16 mg./100 g. During the addition of the large volume necessary, there was probably some fading of the dye during titration, which would make this value higher than the true content. In this one case of experimental cataract there was, therefore, a very marked reduction of ascorbic acid in the apparently normal cornea and a complete absence of ascorbic acid in the aqueous humour and lens.

DISCUSSION

These experiments show that a considerable concentration of ascorbic acid is present in the cornea as a whole and that within the tissue there is an uneven distribution. The concentration in the stroma is about equal to that in the aqueous humour; the concentration in the corneal epithelium is much higher. Friedenwald *et al.* (1943) have produced evidence that ascorbic acid reaches the aqueous humour from the ciliary body. If we consider that the flow of ascorbic acid is in this direction, it seems reasonable to consider the aqueous humour as the source of the ascorbic acid in the cornea. This implies that the corneal endothelium is permeable to ascorbic acid and that the epithelial cells can maintain a higher concentration of ascorbic acid than the corneal stroma. Some evidence in favour of this view has been obtained. The concentration of ascorbic acid in the epithelium was maintained against a lowered concentration in the aqueous humour and ascorbic acid did not diffuse at all freely from the cells into Ringer's solution. On the other hand, the corneal endothelium seemed to be slightly permeable to ascorbic acid in either direction. These facts seem more in accord with the view that the corneal epithelium is able to concentrate ascorbic acid than that the epithelial cells are the source of the ascorbic acid present in the corneal stroma and aqueous humour.

SUMMARY

1. There is a high concentration of ascorbic acid in the ox and rabbit cornea.

2. The concentration is greatest in the corneal epithelium and the concentration in the corneal stroma is about equal to that in the aqueous humour.

3. The corneal endothelium is permeable to ascorbic acid.

4. The concentration of ascorbic acid in the cornea falls after injury with $\beta\beta'$-dichlorodiethyl sulphide and was found to be very low in one case of cataract in a rabbit.

The experiments in which $\beta\beta'$-dichlorodiethyl sulphide was used are reported with the permission of the Ministry of Supply.

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The Manometric Determination of Formic Acid

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In spite of much effort there is no wholly satisfactory method for the estimation of small amounts of formic acid. Most of the methods that have been published depend on the fact that this acid is a volatile reducing agent, but its volatility is rather low and the specific estimation of a reducing agent in biological material is notoriously difficult. Two methods that do not depend on these properties have been described. One involves the liberation of carbon monoxide on treatment with strong sulphuric acid; it has been much used for the recognition of formic acid, but it has not been adapted for convenient analytical use. The other involves the reduction to formaldehyde by magnesium (Fenton & Sisson, 1908; Droller, 1932) and the colorimetric estimation of the formaldehyde. This method appears in several text-books (e.g. Snell & Snell, 1937), but in my hands it has proved unsatisfactory and Pickett, Ley & Zygmuntowicz (1944) have likewise been unsuccessful with it.

Mixtures of volatile acids have been analyzed by collecting and titrating separately several fractions of distillate. The rates of distillation of the individual acids are known so that, by solving a series of simultaneous equations, the composition of mixtures of three or four acids can be ascertained (Hillig & Knudsen, 1942; McClendon, 1944; McNair, 1933). Formic acid distils more slowly than other unsubstituted fatty acids, but it is not clearly differentiated from some of the substituted acids. Distillation has, therefore, generally been used simply as a means of preliminary separation from other components of the mixture. The same result has also been achieved by extracting the formic acid from aqueous solution with an immiscible solvent. This procedure is especially suitable when the effects of acid distillation must be avoided as, for example, when the course of an acid hydrolysis is being followed (Miles & Pirie, 1939) or when free formic acid is being estimated in the presence of components that would give rise to formic acid during acid distillation (Claren, 1942). The method is, however, inconvenient, for no solvent is known with a very favourable partition coefficient and with most solvents the extraction becomes less efficient as it becomes more complete.

Mercuric salts are relatively specific oxidizing agents and they have been most commonly used in the final stage of the estimation, but a wide range of other agents has also been proposed. Among these permanganate (Klein, 1887), bromine (Joseph, 1910) and chromic acid (Tsiropinas, 1917) may be mentioned. These agents have been used in work more recent than that quoted and the reactions have been followed in a wide variety of ways, but all have been severely condemned as relatively unspecific. Stanier & Massart (1935) claim a greater specificity for periodate and Pickett *et al.* (1944) find that ceric sulphate, under defined conditions in which the action is followed by estimation of the CO_2 produced, has a specificity similar to that of mercuric salts. If small amounts of formic acid are being estimated, this ceric method would appear to be the most suitable of those already published.

The early literature on the use of mercuric salts for the oxidation was surveyed by Fincke (1913), and he defined the conditions necessary for quantitative oxidation and listed most of the substances that are liable to interfere with the reaction. Fincke weighed the mercurous chloride formed as a result of the action and this technique has been used by Auerbach & Zeglin (1922) and others, although the physical properties of mercurous chloride make it difficult to manipulate small amounts quantitatively. It has