their vision becomes much poorer, and hence they are very loath to continue this treatment. Curiously enough, it seems to me that it is the patients with pseudo-glaucoma who object particularly to the use of pilocarpin because it makes them so blind.

Dr. Brown's observation is very interesting, because of the curious optic nerve changes observed by those of us who were privileged to follow the epidemic of wood-alcohol blindness which occurred in New York some years ago; in those cases we found exactly this same type of optic nerve change, and this suggested to me that there must be some other condition than pure glaucoma to explain this process.

THE RÔLE OF ASCORBIC ACID (VITAMIN C) IN THE SECRETION OF THE INTRA-OCULAR FLUID*

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In a previous paper[1] certain components of the secretory mechanism in the ciliary body were described, and a theory was formulated to explain their interaction. The present study represents a continuation of the same investigation.

Ascorbic acid (vitamin C) is a substance derived from the blood, but present in the intra-ocular fluid in concentrations appreciably higher than in the blood.[2] Energy must, therefore, be expended by the tissues in order to maintain this difference in concentration. The previous study revealed that energy was available in the ciliary processes for such

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secretory work. We were interested, therefore, in ascertaining whether the conceptions of the secretory mechanism previously formulated were adequate to explain also the intra-ocular secretion of ascorbic acid. The answer to this question constituted the initial objective of the present study. It was soon found, however, that ascorbic acid was not merely passively acted upon by the secretory mechanism, but that, in fact, the ascorbic acid system constituted an integral part of the secretory mechanism, and the mechanism of intra-ocular secretion of ascorbic acid was adequately explained only when it was found to be a simple consequence of the rôle played by this substance in the energy transformation of secretion. In the present communication we shall deal only with the active rôle of the ascorbic acid system in the secretory mechanism. The consequences of the findings here reported in relation to the mechanism of intra-ocular secretion of ascorbic acid will be dealt with in another article.

In the paper previously referred to it was shown that the energy of secretion could be derived from a difference in oxidation-reduction potential between the epithelium and the stroma of the ciliary body. This difference in potential was found to be the result of a difference in the respiratory enzymes of the two tissues. The interaction of these two tissues through the intermediation of a barrier or membrane that exists between them could result in the transformation of this available energy into work. This barrier was shown to possess the characteristics of membrane charge and reactivity requisite for this function of mediation between stroma and epithelium (fig. 1).

Up to this point in the analysis the stroma of the ciliary body has been regarded as a functional unit, and, in fact, the study of the oxidation-reduction potential revealed no differences between its component parts—capillary endothelium, stroma cells, and interstitial tissue. The oxidation-reduction potential is plainly generated by the action of respiratory enzymes upon their metabolic substrates. It follows

that the well-stabilized potential and large reducing capacity which were found to characterize the interstitial tissue of the ciliary stroma must be due to the presence in the extracellular fluid either of respiratory enzymes and metabolites, or of some reversibly oxidizable substance or substances that can diffuse into and out of the cells, and hence maintain in the extracellular spaces the potentials generated within the cells. Our first problem, therefore, consists in making a study of the reducing system in the interstitial tissue of the ciliary stroma. As in the previous paper, the continuity of the argument will

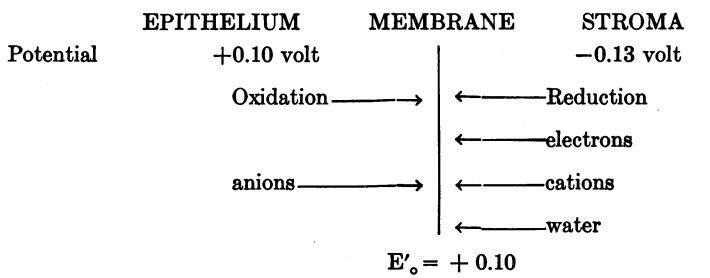

Fig. 1.—Schematic representation of the secretory system.

be maintained in the large type of the text, whereas experimental technique and experimental protocols will be printed in smaller type.

I. THE REDUCING SUBSTANCES IN THE CILIARY STROMA

(a) *Histologic Studies*

Szent Györgyi, in his studies on the reducing substances in the adrenal gland, studies that led him eventually to the isolation and identification of ascorbic acid, developed a histologic technique for the demonstration and localization of reducing substances in the tissues. These techniques have been modified, and their specificity as regards ascorbic acid has been increased by Giroud and Leblond.[3] We have applied these techniques to the ocular tissues.

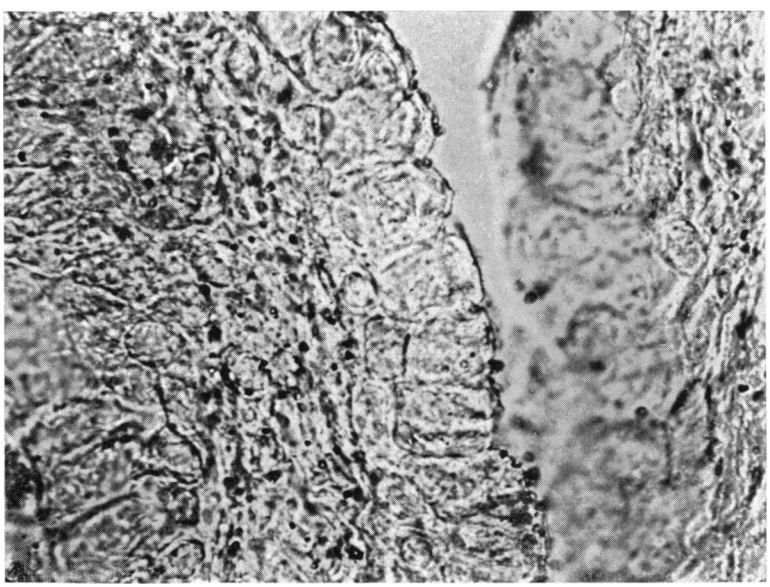

Fig. 2.—Silver granules in the stroma.

The iris and ciliary body are removed from the eye of an albino rabbit and cut up into slices, as described in the previous paper. Portions of the tissue are placed in a 5 to 10 per cent. solution of silver nitrate, either neutral or acid (about pH 4), the reaction taking place in the dark. After from five to ten minutes the tissue is washed in distilled water without exposure to light, and transferred to a solution of 10 per cent. sodium thiosulphate ($Na_2S_2O_3$), which removes all the silver chlorid and other unreduced silver. The tissue may now be exposed to light without affecting the reaction. It is dehydrated and imbedded in paraffin. Unstained or stained sections may be examined.

In this technique metallic silver in the form of black granules is deposited in the tissue wherever substances capable of reducing silver nitrate existed. On microscopic examination black granules are found in the stroma of the ciliary processes. The granules do not accumulate in the capillary walls, but are distributed fairly uniformly in the extravascular tissue, stroma cells, and interstitial tissue. Only a few granules are found in the epithelium or in the ciliary muscle (fig. 2). Many granules are found in the iris, being most concentrated near the anterior surface and about the vessels. The silver granules are found in tissue treated with silver nitrate in both neutral solution and at pH 4. Previous studies have shown that the dehydrogenase enzymes of the ciliary stroma are inactive at the latter pH. Hence the reduction of the silver is accomplished by active reducing substances present in the tissues, and not by normally inactive metabolites that have been activated by enzymes.

To summarize: The interstitial tissue of the ciliary processes contains an active reducing substance capable of reaction with silver nitrate in an acid medium. The implication that a portion of this reducing substance may be ascorbic acid is obvious, but the identification of this acid can be based only on more specific tests. It is not to be implied, however, that *all* the reducing substances in the ciliary processes are ascorbic acid. The reason for assuming that some other reducing substance must also be present is as follows:

It has been shown that the normal potential of ascorbic acid at physiologic pH is approximately +0.04 volt, whereas the potential of the stroma is −0.13. At the latter potential not more than one part in 100,000 of the ascorbic acid present could be oxidized. The fact that oxidized ascorbic acid (dehydroascorbic acid) undergoes rapid spontaneous decomposition at this pH does not account for the low concentration of the oxidant, for if an appreciable portion of the ascorbic acid were being oxidized,—say 1 per cent. a minute,—and if the oxidant decomposed at such a rate that one-half disappeared a minute, a steady state would be achieved in which approximately 1 per cent. of the ascorbic acid was present in oxidized form. Since the potential of the tissues characterizes a steady state in which oxidation and reduction are taking place continuously, it follows that, if free ascorbic acid is present in the stroma, and if ascorbic acid takes a part in the oxidation-reduction reaction, it must be accompanied by some other reducing substance whose normal potential is closer to the potential of the stroma—a substance capable of rapidly reducing any dehydroascorbic acid that may be formed.

(*b*) *Chemical Studies*

The most specific test for the presence of ascorbic acid lies in the prevention of scurvy in guinea pigs, and most of the reducing substance in the aqueous of cows' eyes has been shown to be ascorbic acid by this means. In spite of the high concentration of reducing substance in the ciliary body, the volume of this tissue is so small that approximately 1,000 rabbit ciliary bodies would have to be extracted in order to provide sufficient material for one complete feeding test. The chemical technique at present available for the identification of ascorbic acid in tissue extracts leaves much to be desired. Those that are potentially applicable to the minute quantities available in the ocular tissues are dependent essentially on the reducing ability of this substance. Many other substances are, however, likely to be present in tissue extracts,

and these may also possess reducing ability. Some of these possible contaminants, such as the sulfhydryl compounds, may be distinguished by special reactions, but in many others no such reactions are known. For the most part, reliance has been placed on the rate of reaction and control of pH—ascorbic acid tending to react more rapidly than any of the known contaminants at certain pH.

TABLE I.—CONCENTRATION OF REDUCING SUBSTANCES IN THE CILIARY BODY

In milli-equivalents (meq.) per 1,000 gm. of fresh tissue (1 meq. ascorbic acid = 88 mg. ascorbic acid)

	Chemical Titration with			*Electrometric Titration with Ferricyanide*	
	Methylene Blue	*2, 6-Dichlorophenol Indophenol*	*Iodin*	*Ascorbic Acid*	*Total Titration*
Rabbit[1]	2.3 (3)	2.7 (3)	4.0 (2)	2.0 (3)	4.0 (3)
Pig[1]	0.6 (2)	..	2.7 (2)	..	..
Cattle[2]	1.9 (2)	2.1 (2)	3.7 (3)	2.0 (2)	3.0 (2)
Guinea pig[1]					
Vitamin C deficient	..	0.5 (1)	3.4 (1)	0.7 (1)	..
Vitamin C saturated	..	1.6 (2)	4.5 (2)	1.0 (1)	3.3 (1)
Cat[1]	1.2 (2)	2.2 (1)	..	1.9 (1)	3.6 (1)

[1] Ciliary body + iris.
[2] Isolated ciliary processes.
The numbers in ( ) indicate the number of experiments.

Using these criteria, it is found that the total reducing substances (iodin titration)[4, 5] of the ciliary body and iris of rabbits amount to 4 milli-equivalents (per 1,000 grams of fresh tissue), of which 50 to 60 per cent. may be titrated with methylene blue* and identified provisionally as ascorbic acid (Table I). Only very slightly different values have been obtained by titration with 2,6-dichlorophenol indophenol at pH below 5,† a method that is more suitable for small amounts of tissue extracts and low concentrations of reducing substance than is the methylene-blue method. Roughly sim-

* After the procedure of Martini and Bonsignore.[6]
† After the procedure of Harris.[7]

ilar figures are obtained on extracts of the isolated ciliary processes of cows. Pigs' ciliary body and iris show somewhat lower methylene-blue values than the respective tissues of rabbits and cows, but the difference between iodin and methylene-blue figures amounts also in pigs to roughly 2 milli-equivalents.*

Technique of tissue extraction and titration: The tissues, taken from the animals' eyes as fresh as possible, were frozen immediately in a centrifuge tube of known weight which had been immersed in a freezing mixture; then the tissues, in frozen state, were ground up in the centrifuge tube by a glass rod and the exact weight determined. The tissue was then extracted for several minutes in about three times its weight of 4 to 8 per cent. sulfosalicylic acid and then centrifuged. In some of the earlier experiments 8 per cent. trichloracetic acid had been used instead of the sulfosalicylic acid, and the tissues were ground up with sand in a mortar. Later we adopted the use of the frozen tissue extraction and of the sulfosalicylic acid as the standard procedure, because they meet the requirements of the titration of very small tissue amounts (*e. g.*, of guinea pigs' eyes), and of the electrometric titration more conveniently.

Iodin titration: To 0.2—0.5 c.c. of extract, 0.1 c.c. of 1 per cent. potassium iodid (KI) and two drops of 1 per cent. starch solution were added, and then, from a microburette, the mixture was titrated with a N/1000 potassium iodate solution (KIO_3) until the first blue color appeared and persisted. The iodate was standardized against a freshly prepared solution of pure ascorbic acid.

Methylene-blue titration: 0.5 c.c. of the extract was neutralized and buffered with 0.4 N NaOH and 0.4 N acetate buffer to a pH of 4.9 (empirically determined with pH indicator dyes and occasionally checked electrometrically). Then 0.1 c.c. of a saturated sodium thiosulphate solution was added (in order to stabilize the leukoform of methylene-blue during the titration). The titration was carried out from a microburette with a methylene-blue solution 1:10,000 in acetate buffer pH 4.9 under intense illumination of the titration vessel. The illumination was done by a 150-watt electric bulb from below, with an arrangement similar to that devised by Lund and Lieck.[8]

The end-point of the titration was determined by comparison

* A possible source of error in the pigs' tissue titration has been introduced by the fact that the material from the pigs' slaughterhouse could not be obtained quite so fresh as the tissue of the cows and of the laboratory animals.

with an equal amount of water to which 0.04 c.c. of the methylene-blue solution had been added. From this titration value 0.04 c.c. had to be subtracted. Special care had to be taken to avoid spontaneous reoxidation of the formed leuko-methylene blue during the titration, and to avoid a possible error by disregard of the actual pH, the importance of which has been stressed by Neuweiler.[9] In order to meet the first point, we used titration vessels of a small diameter (1–2 cm.) so that the contact surface with the air was relatively small, and we cooled the bulb container and the bulb by ventilation with a stream of air. The titration was carried out as fast as possible (one to two minutes). The possible influence of the pH was controlled in addition to the buffer procedure by doing the standardization of the methylene blue with pure ascorbic acid dissolved in a sample of the tissue extract itself which had been neutralized and buffered in the usual manner.

The dichlorophenol-indophenol-titration in acid solution (after Harris,[7] *loc. cit.*) does not require any special comment. The standardization of the approximately N/1000 dye solution was also made with pure ascorbic acid.

The titration of the ciliary body and iris of guinea pigs was performed with special micropipettes and in microtest tubes, as described below for the aqueous of guinea pigs.

The specificity of these tests leaves much to be desired. Ball[10] has shown that solutions containing ascorbic acid and dehydroascorbic acid in definite proportions at definite pH exhibit characteristic potentials that may be measured with appropriate electrodes, etc. It follows that if, under nitrogen and at fixed pH, small amounts of an oxidant (ferricyanid) are added successively to a solution initially containing ascorbic acid, and the potential measured after successive additions, a relation may be established between the potential recorded and the percentage of ascorbic acid oxidized which is highly characteristic for this substance. Ball has used this potentiometric titration curve to identify the redox material in orange juice as ascorbic acid. He stresses the importance of doing the electrometric titration at pH below 5, because the dehydroascorbic acid at higher pH becomes increasingly unstable. For a definite identification of ascorbic

acid, therefore, the electrometric titration at a low pH is to be preferred. This precaution, which has not been observed in a number of earlier reports dealing with the electrometric determination of ascorbic acid in biologic material, was followed carefully by us for the sake of the definite identification of the reducing substances in our material. By using suitably small titration chambers and microburettes it is possible to adapt this procedure to the titration of extracts of iris and ciliary body of a single rabbit eye.

A protein-free extract of the tissue was prepared as previously outlined. Frequently sufficient extract was prepared to make possible parallel chemical titration (methylene blue, dichlorophenol indophenol, and iodin titrations) and potentiometric titration. The sample to be titrated potentiometrically was brought to pH 4.62 with acetate buffer, $\frac{N}{1000}$ potassium ferricyanid, and $\frac{N}{10,000}$ methylene blue or thionin being added to act as mediators. The solution was placed in a titration chamber,[6] and deaërated by bubbling through a steady stream of oxygen-free nitrogen. Freshly gold-plated platinum electrodes were used, also a calomel half cell and saturated KCl salt bridge. The oxidant ($K_3Fe(CN)_6$), dissolved in acetate buffer and deaërated, is added from a microburette. Both tissue extracts and solutions of ascorbic acid used as controls come slowly to equilibrium with the electrodes. We have taken as the end-point of the potential drift that potential at which the drift was less than 0.1 millivolt a minute.

Using known solutions of ascorbic acid as controls, we have been able to repeat the measurements described by Ball.[10] The midpoint of the titration curve (50 per cent. oxidation) was found to agree with that predicted by Ball to within ± 5 millivolts. The titration curve obtained with an extract of cow's ciliary body or rabbit's ciliary body and iris shows one portion to which the theoretic curve for ascorbic acid can be fitted with equal accuracy (fig. 3). By this means the identification of ascorbic acid as one component of the redox system in the tissue extract may be assured. A comparison of the chemical and potentiometric titrations of the same sample of tissue extract reveals excellent agreement between the

methylene-blue titration and that part of the potentiometric titration which can be attributed to ascorbic acid. Hence the specificity of the methylene-blue titration as a measure of ascorbic acid in these extracts is established.

These titrations reveal the presence, in the extract, of at least one other redox system more negative than the ascorbic acid system. The nature of this substance or substances is the subject of current investigation. In addition, it may be observed that the total reducing substance as titrated with iodin is usually found to be slightly greater than the total

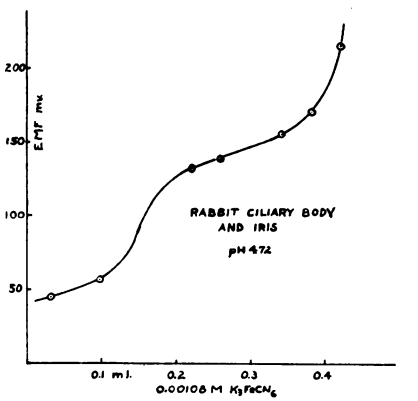

Fig. 3.—Oxidative titration of tissue extract.

reducing substance as titrated with ferricyanid. This suggests that a small amount of some redox system more positive than the ferricyanid system may be present.

To summarize: Protein-free extracts of the ciliary processes of cows, and of the ciliary body and iris of rabbits, contain 4 milli-equivalents of reducing substances, of which from 50 to 60 per cent. may be identified as ascorbic acid. The remainder consists mainly of an as yet unidentified substance or group of substances which react more sluggishly than does ascorbic acid, and which are titrated at potentials considerably more negative than ascorbic acid. For the present we shall refer to this as the second interstitial mediator.

II. Ascorbic Acid in the Mechanism of Secretion

In the previous section we showed how the theory of the secretory mechanism led us to seek for reducing substances in the stroma of the ciliary processes, and how, of the reducing substances found, one component could be identified as ascorbic acid. We may now inquire whether or not the ascorbic acid actually plays a rôle in the secretory mechanism. In order to determine this we have sought to discover what changes, if any, take place in the secretory mechanism on deprivation of vitamin C. Since rabbits are not susceptible to scurvy, it became necessary to extend our investigation to guinea pigs.

(*a*) *Reducing Substances in the Ocular Tissues of Guinea Pigs*

As a preliminary step to the study of the effect of vitamin C deprivation on the secretory mechanism, it was first necessary to study the effect of diet on the ascorbic-acid content of the ciliary body and aqueous.

In estimating the redox material in the iris and ciliary body of guinea pigs, it was found necessary to combine tissue from 10 to 18 eyes. Extraction and titration were performed as previously outlined. Estimate of the concentration of reducing substance in the aqueous can be made on fluid obtained from a single guinea pig eye as follows: The aqueous is obtained by corneal puncture with a fine glass capillary. From 20 to 50 c.mm. may be withdrawn. One to three drops of fluid are expelled from the pipette into a minute test-tube (internal dimensions, 3 x 20 mm.). One drop of 10 per cent. acetic acid solution is added from a similar pipette, and a glass bead is introduced to facilitate mixing, which is accomplished by gently tapping the tube with a finger-nail. From another similar pipette successive drops of a solution of 2,6-dichlorophenol indophenol (about $\frac{N}{3000}$) are added until a permanent color is obtained. The drop size of the initial pipette and the dye solution are calibrated and standardized by titrating a standard solution of ascorbic acid in the same way. With care the method may be made sensitive to 0.5 gamma ascorbic acid, the error of method being not higher than ± 10 per cent.

It was found that adult well-nourished animals that have been fed on a diet containing abundant vitamin C have reducing substance (titratable with indophenol) in the aqueous amounting to between 7 and 20 mg. per cent. These animals likewise have (iodin) reducing substance in the ciliary body and iris in a concentration of about 3 to 4 milli-equivalents. Of this, approximately 30 to 40 per cent. is titratable with 2,6-dichlorophenol indophenol, and by electrometric titration can be shown to be ascorbic acid. When these animals are placed upon a vitamin C free diet (dry hay, dry oats, water, and cod liver oil), the reducing substance in the aqueous rapidly decreases, reaching immeasurably small concentrations in from four to six days. The disappearance of titratable reducing substance from the aqueous is accompanied by a marked decrease of the dichlorophenol indophenol titratable fraction from the ciliary body and iris. It is of particular interest that the decrease of ascorbic acid in the ocular tissues takes place before any constitutional symptoms of scurvy develop. The animals still appear to be well nourished and lively, and have lost only from 2 to 5 per cent. of their body weight.

When similar studies are made in young guinea pigs weighing approximately 200 grams, it is found that the disappearance of ascorbic acid from the ocular tissues takes place even more rapidly. In from twenty-four to forty-eight hours after the last vitamin C containing meal, no titratable reducing substance is to be found in the aqueous of these animals. It is evident that these young guinea pigs are deficient in ability to store ascorbic acid in their ocular tissues. Nevertheless, they do not develop clinical symptoms of scurvy until they have been on the deficient diet for from ten to fourteen days, and death occurs in three to four weeks. The lability of the ascorbic-acid content in guinea pigs' eyes explains the widely varying reports of different previous investigators as to the "normal" concentration of ascorbic acid in the aqueous of these animals. In order regularly to obtain animals with high

ascorbic-acid content of the ocular tissues, special precaution must be taken to insure a high intake of vitamin C during the days and also the hours preceding an experimental observation.

In both adult and young animals that have been deprived of vitamin C, the intravenous injection of ascorbic acid is promptly followed by the reappearance of reducing substance in the aqueous. The silver staining, which gives a negative result in the ciliary body of scorbutic guinea pigs, becomes promptly positive again after the intravenous injection of ascorbic acid.

To summarize: These experiments have made it clear that ascorbic acid is a normal component of the ocular tissues in the guinea pig as in the rabbit, and, further, that ascorbic acid can be eliminated from the ocular tissues of guinea pigs by simple dietary restrictions without simultaneously producing such severe general malnutrition as to complicate the experimental picture. Finally, these results show that indophenol titration of the aqueous furnishes a satisfactory test as to the state of depletion of the ocular tissues.

(*b*) *Oxidation-Reduction Potential*

In testing the potential of the ciliary tissues the technique reported in the previous paper was used with minor modifications. In general, aqueous was removed from one eye for indophenol titration, whereas the other eye was used for potential measurements. When it was desired to use both eyes of the same animal for potential measurements, *e. g.*, before and after intravenous injection of ascorbic acid, a cage mate similar in size and age to the original animal was used for titration control.

In making preparations for supravital staining and observation, it was found that the iris and ciliary body of the guinea pig could not readily be stripped out of the eye, as was the case with the rabbit. Hence, after equatorial section of the eyeball and the removal of lens and vitreous, the anterior segment—cornea, anterior

portion of the sclera, iris, and ciliary body—was cut up into four to six wedge-shaped slices, and placed on glass slides with the intra-ocular surface up. The ciliary processes of the guinea pigs are relatively short and thick, and are not readily lapped over the edge of such a preparation. It was found best, therefore, when testing the epithelium with some dye, to place a minute drop of the dye on the moist posterior surface of the iris, whence it spreads readily to the anterior tips of the ciliary processes. In this way quite sharp preparations could be obtained.

TABLE II.—OXIDATION-REDUCTION POTENTIAL IN CILIARY BODY

(Volt; pH 7.4)

	Air		*NaCN*	*Nitrogen*
	Epithelium	*Stroma*	*Epithelium or Stroma*	*Epithelium or Stroma*
Rabbit	+0.100	−0.130	−0.200	−0.290
Guinea pig:				
Vitamin C deficient	+0.175	−0.260	−0.275	−0.290
Vitamin C saturated	+0.100	−0.160	−0.200	−0.290
Cat	±0	−0.270	..	−0.370

The results of these tests presented in Table II show that the potential of the ciliary tissues in the normal guinea pig is closely similar to that found in rabbits. When ascorbic acid is absent from the tissues, the aërobic potential of the epithelium becomes more strongly positive, and that of the stroma more negative. The increase in negativity of the stroma cannot be attributed to the addition to the tissue of some new reducing system, since the anaërobic potential is unchanged. When ascorbic acid is reintroduced into the system by intravenous injection, the potentials of the ciliary tissues immediately revert to the normal level. In the time required to make the injection, remove an eye under ether anesthesia and expose the tissue to the test dye—two to three minutes—and the transformation is complete. The rapidity of this change would indicate that ascorbic acid itself, rather than some other substance that might become available in-

directly as the result of the intravenous injection of ascorbic acid, is directly responsible for the change.

The simultaneous rise in potential of the stroma and fall in potential of the epithelium is evidence that ascorbic acid facilitates the oxidation-reduction interaction between epithelium and stroma, and hence that ascorbic acid acts as a mediator between these two tissues. It follows also that under normal conditions such interaction between the two tissues is actually taking place. This feature of the secretory system which was postulated in the previous paper is now supported by direct experimental evidence.

In Table II it will be seen that, under Cyanid Poisoning, the potential of the stroma is higher when ascorbic acid is present. Since the increased rate of oxidation which this denotes cannot be attributed to oxidation arising in the epithelium, for the two tissues now have equal potential, it follows that in the tissues ascorbic acid reacts more rapidly with atmospheric oxygen than do the other reducing substances that remain even when ascorbic acid is absent. The very small difference between aërobic and anaërobic potential in the stroma in the absence of ascorbic acid indicates that without ascorbic acid the stroma possesses essentially an anaërobic respiratory system. The still smaller potential difference between cyanid and nitrogen asphyxia in the vitamin C deficient animal may be due entirely to the reaction of the test indicator (safranin) with atmospheric oxygen. The oxidation which goes on normally in the ciliary stroma when ascorbic acid is present can thus be completely accounted for by the oxidative contribution of the epithelium, plus the aërobic oxidation of ascorbic acid. The nature of the catalyst responsible for this aërobic oxidation is unknown. On the other hand, the fact that in the vitamin C deficient animal the introduction of cyanid causes a slight decrease in potential indicates that some oxidation of the stroma by cytochrome oxidase occurs even in the relative absence of ascorbic acid. The question arises, therefore, as to whether the stroma contains small amounts of

such oxidase, or whether, even in the vitamin C deficient animals, some small amount of interaction between stroma and epithelium may still continue, mediated by some other less efficient component of the reducing substances of the stroma, or by traces of ascorbic acid still remaining.

In the previous paper[1] the employment of the usual indophenol reagent (Nadi reagent) in demonstrating the presence of cytochrome oxidase (indophenol oxidase) in the epithelium was reported. With this reagent no evidence was found for the existence of this enzyme in the stroma, and it was concluded that the stroma lacked cytochrome oxidase. It is possible, however, that at a strongly negative potential the reaction might be negative, even though the enzyme were present in small amount. In order to test this possibility, the following experiment was performed:

A sample of iris and ciliary body freshly removed from a rabbit was placed in Ringer's solution with $\frac{N}{1000}$ ferricyanid. After five minutes the yellow color of the ferricyanid could be observed in the stroma. The tissue was then washed in Ringer's solution until the ferricyanid color had been removed, and then was placed in the indophenol reagent. The characteristic color of indophenol blue appeared promptly in the epithelium, indicating that the treatment had not inactivated the oxidase. Nevertheless, no color beyond that faint blue which appears also in the bathing fluid developed in the stroma.

This experiment indicates that the stroma contains no cytochrome oxidase, and hence that the slight cyanid effect on the stroma of vitamin C deficient animals is probably to be attributed to a slight residual interaction between stroma and epithelium.

One other feature of the potential measurements requires comment. In the rabbit and in the guinea pig, with ample ascorbic acid, the potentials of the tissues were found to be quite stable, remaining unchanged in tissue fragments in Ringer's solution for one-half hour to one hour. In the vitamin C deficient guinea pig the strong reducing potential of

the stroma begins to disappear shortly after the tissue has been removed from the body. In fresh tissue the reduction of several of the safranin dyes was readily demonstrated. A similar piece of tissue, after being in Ringer's solution for fifteen minutes, may be unable to reduce methylene blue.

A possible explanation of this phenomenon may be offered in that, if the ionic electrical current in the tissues is absent or very feeble, anions would more readily escape from the ciliary stroma. It is not unlikely that in this way necessary metabolites and mediators might be lost from the stroma.

To summarize: The oxidation-reduction potential of the ciliary tissues is profoundly affected by the presence or absence of ascorbic acid. In the absence of ascorbic acid, the respiration of the stroma is essentially anaërobic. In the presence of ascorbic acid, oxidation of the stroma by molecular oxygen takes place. Part of this oxidation is attributable to aërobic oxidation of ascorbic acid, the remainder to the effect of enzymes which are capable of being inactivated by cyanid. These enzymes are present only in the epithelium. It is concluded, therefore, that oxidative-reductive interaction between epithelium and stroma takes places, and that ascorbic acid acts as a mediator that facilitates this interaction.

(c) *Irreciprocal Permeability to Dyes*

In the previous paper[1] it was pointed out that if oxidative-reductive interaction should take place between epithelium and stroma, an electrical current would be generated that would manifest itself in the movement of ions, cations being transferred from stroma to epithelium, and anions being transferred from epithelium to stroma. By introducing colored ions, *i. e.*, dyes, into the tissue, this iontophoresis is rendered observable. We may now apply this same technique in the presence and absence of ascorbic acid.

In the experiments of this type reported in the previous paper, the most striking illustration of ionic transfer was observed in the case of basic dyes. A sublethal dose of crystal

violet was injected intravenously into a rabbit. Immediately after completing the injection, one eye was enucleated and a portion of the ciliary body was placed on a slide for microscopic examination. It was found that almost all the dye in the ciliary body was already concentrated in the epithelium. The whole procedure can be performed in less than two minutes, but it cannot be conducted so quickly as to enable one to see any considerable portion of the crystal violet that is still in transit in the stroma. The same experiment performed on a normal guinea pig not deficient in vitamin C yields similar results. When this experiment is done on guinea pigs deficient in vitamin C, the dye is found, on the first examination, to be mainly in the stroma. In order to obtain the same distribution of the dye in the deficient as in the normal animal, one must wait ten to fifteen minutes after the intravenous injection of the dye before removing the tissues for examination. If ascorbic acid is injected intravenously immediately before the crystal violet, the normal picture of rapid accumulation of the dye in the epithelium is obtained. That the slow penetration of crystal violet in the vitamin C deficient tissues is not due to any change in the permeability of the stroma-epithelium barrier may be demonstrated as follows:

Two freshly excised pieces of ciliary body, one from a normal and one from a vitamin C deficient guinea pig are placed on a slide, and a minute drop of crystal violet solution is brought into contact with the epithelium of each specimen. The epithelium rapidly takes up the stain. If these tissues are now observed aërobically, the dye is seen to remain in the epithelium, but if they are observed under nitrogen or cyanid asphyxia (under these conditions the ionic electric current in the tissues is inhibited), the dye slowly penetrates equally into the stroma in the two specimens.

To summarize: The ionic electric current, as demonstrated by the movement of basic dyes in the tissue, is greatly reduced in intensity in the absence of ascorbic acid, but is not entirely absent even in extreme scurvy. The persistence of

a small residual interaction between stroma and epithelium, even in vitamin C deficient animals, is thus confirmed.

(*d*) *Transfer of Water*

In the previous paper[1] it was shown that the passage of an ionic electrical current through the ocular tissues was accompanied by the transfer of water, and that the ionic electrical current normally present in the tissues has the proper direction to carry water from stroma to epithelium, *i. e.*, in the direction of the normal secretory flow. No quantitative relations could, however, be asserted, and no conclusions were drawn as to whether the postulated mechanism accounted for a trivial or an important component of the water transfer. The possibility of inactivating and reactivating the secretory mechanism by deprivation or administration of ascorbic acid enables us to test directly the contribution of this mechanism in respect to the transfer of water.

Owing to the small volume of guinea pig eyes it was necessary to construct a special manometer with which to measure the intraocular pressure with minimal displacement of fluid being required to register a reading. The apparatus which was prepared (see fig. 4) is a modification of the familiar compensating manometer. The diameter of the narrow portions of glass capillary is 0.25 mm. The remaining glass connections have an internal diameter of 1 mm. The lower portions of the instrument are filled with mercury. By turning the screw clamp over the rubber tube, the position of the mercury meniscus can be adjusted to any desired level. In practice, the mercury is first made to fill the manometer up to the needle. The tip of the needle is then immersed in a drop of Ringer's solution, and the mercury is allowed to drop back to the mark I, the tube from I to the tip of the needle being filled with Ringer's fluid. The stopcock is now turned so as to prevent further movement of the mercury, and the needle is introduced into the anterior chamber of a guinea pig (intraperitoneal urethane anesthesia, 1.25 gm. per kilo plus conjunctival instillation of one-half per cent. pontocaine).

When the apparatus has been fixed rigidly in place, the stopcock is turned so as to be open all three ways. If the meniscus tends to move from position I, it is brought back by a turn of the screw

clamp. The pressure is measured by the position of the free meniscus in CD; the level corresponding to zero pressure is determined at the end of the experiment without changing the tilt of the manometer. After the pressure has been recorded for from five to ten minutes, the screw clamp is turned so as to bring the meniscus down to position II. The volume of fluid withdrawn from the eye by this procedure is the volume of the tube between I and II,

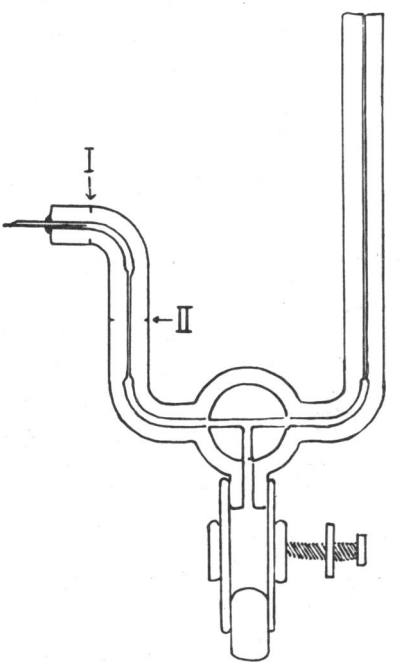

Fig. 4.—Manometer for use on guinea pigs.

and amounts to 17 c.mm. This is approximately one-third to one-half the volume of the anterior chamber in an adult guinea pig, and its withdrawal results in a drop of from 9 to 16 mm. Hg in intra-ocular pressure. The meniscus is now kept at position II by continuous manual adjustments of the screw clamp, and the return toward normal of the intra-ocular pressure can be observed (fig. 5).

In each experiment a sample of aqueous for dichlorophenol indophenol titration was withdrawn from one eye immediately prior to

inserting the manometer cannula in the other eye. If ascorbic acid was injected intravenously during the course of the experiment, a similar sample was withdrawn from the second eye at the end of the experiment. Figure 5 shows the result of a series of experiments of this type.

The normal intra-ocular pressure of guinea pigs under the conditions of the experiments was found to be between 15 and 25 mm. Hg. No significant differences in pressure were found between normal and vitamin C deficient animals. In normal

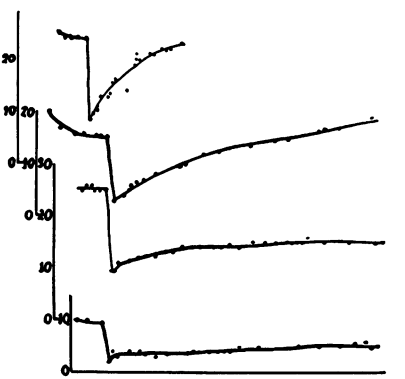

Fig. 5.—Recovery of intra-ocular pressure after withdrawal of aqueous. The two upper curves were obtained on animals saturated with vitamin C, the two lower curves on animals in states of vitamin C deficiency.

animals the withdrawal of intra-ocular fluid and the consequent lowering of intra-ocular pressure were generally followed by a rapid recovery, the pressure reaching the previous normal level in from ten to twenty minutes and generally overshooting that level (reactive hypertonia). In animals deficient in ascorbic acid the return to normal pressure is, by comparison, painfully slow. Most characteristic is the difference in the shape of the recovery curve in the two cases. In the typical normal animal the recovery of intra-ocular pressure begins almost immediately with a steep rise, which gradually flattens out, yielding a curve convex upward in its

whole extent. In the deficient animal the onset of recovery develops slowly, and little change in pressure may take place during the first ten minutes. The rise, when it finally occurs, is a gradual one, almost linear at first, but flattening out as the normal level is approached. The previous norm is rarely overshot, and often not attained.

In this experiment, as in those previously reported, special precautions were required to insure the presence of an effectively high concentration of ascorbic acid in the ocular tissues. Even then, some animals with titratable ascorbic acid in the other eye failed to show a rapid recovery of intra-ocular pressure. Furthermore, it was not possible, by intravenous injection in deficient animals, to bring about an immediate conversion of the pressure curve to the normal picture. It is evident from these experiments that the presence of some other factor, in addition to ascorbic acid, is necessary for the efficient transfer of water. Since the steep curve never occurred in the absence of ascorbic acid, we conclude that ascorbic acid is necessary for the normal transfer of water.

The fact that approximately normal intra-ocular pressure can be maintained in the absence of ascorbic acid is not surprising. The pressure in the eye is maintained by a balance between the activity of the secretory and of the reabsorptive mechanism. We have previously shown[7] that the reabsorptive mechanism is under the control of vasomotor reflexes, and that the reabsorptive mechanism automatically compensates for the increased rate of formation of intra-ocular fluid which results from hyperemia of the intra-ocular tissues, or from increased arterial blood-pressure. The results of the experiments just reported indicate that the reabsorptive mechanism can compensate equally well for a decrease in the rate of formation of the intra-ocular fluid. It is only by testing the rate of formation of intra-ocular fluid at subnormal pressure at which the reabsorptive mechanism is presumably inactivated in both normal and vitamin C deficient animals

that the difference in the rate of formation of the fluid can be demonstrated.

To summarize: The mechanism of secretion of intra-ocular fluid in guinea pigs requires for its normal operation the presence of ascorbic acid in the tissue. When the oxidation-reduction interaction between stroma and epithelium is suppressed by the deprivation of this mediating substance, the rate of transfer of water is greatly reduced. The transfer of electrons, ions, and water across the stroma-epithelium barrier in the ciliary body is thus shown to be a co-ordinated process, and the previously postulated mechanism of secretion is seen to account for all or most of the secretory activity.

COMMENT

In the previous paper[1] studies were reported on the secretory mechanism of the ciliary body of rabbits, and a theory was formulated that was shown to be capable of explaining all the relevant known facts. It would seem wise at this point to review those parts of this theory that were supported by direct experimental evidence and those parts that were postulated in order to complete the theory.

It was shown that a difference in potential between epithelium and stroma in the ciliary body exists. It was shown further that a barrier between stroma and epithelium exists which contains as one of its components a substance capable of reversible oxidation and reduction. It was postulated that oxidation-reduction interaction between stroma and epithelium takes place, the stroma-epithelium barrier acting as mediator between these tissues. It follows from this postulate that electrons and cations may be transferred from stroma to epithelium, and anions from epithelium to stroma. It was found that dyes that were anions, cations, or uncharged molecules penetrated the tissue in the manner predicted. This proved that interaction between stroma and epithelium may take place. In the present paper, experimental evidence was

adduced to show that oxidation of the stroma by the epithelium does in fact take place.

In the previous paper it was demonstrated that the barrier between stroma and epithelium is negatively charged, and that the passage of an ionic electrical current through this membrane was accompanied by a transfer of water by electroendosmosis. It was postulated, therefore, that the secretory movement of water across the membrane was accomplished by the electrical current generated through the interaction of stroma and epithelium.

In the present paper the dependence of the movement of

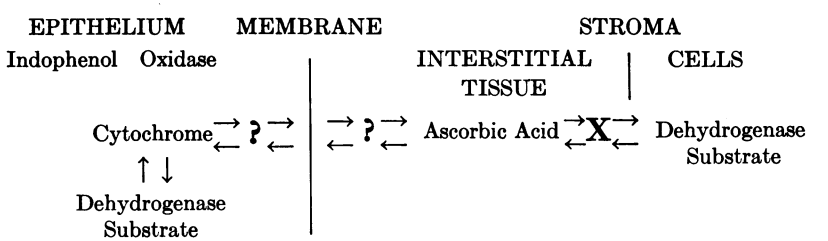

Fig. 6.—Schematic representation of the chemical components of the secretory system.

water into the eye upon the interaction between epithelium and stroma was shown to be directly observable. With these two postulated features replaced by experimental observation, the hypothesis which we have formulated regarding the mechanism of secretion has been completely confirmed. This hypothesis is further expanded (fig. 6). It is shown that the interstitial tissue of the ciliary stroma contains substances capable of reversible oxidation-reduction, which act as mediators in the oxidation-reduction interaction between stroma and epithelium, occupying that portion of the chain of interacting substances between the stroma-epithelium barrier, on the one hand, and the stroma cells, on the other. One of these mediating substances has been identified as ascorbic acid. This appears to be the most readily oxidizable of the interstitial mediators, and hence may provisionally be

assigned the rôle of being the principal substance to be oxidized directly by the stroma-epithelium barrier. With the identification of ascorbic acid and the demonstration of its rôle in the ciliary body, the theory of the secretory mechanism begins to be concerned with substances as well as with their chemical and structural relations. It may be pointed out, however, that ascorbic acid is not always and everywhere a necessary constituent of the secretory mechanism. Even in scorbutic guinea pigs, some slow intra-ocular secretion takes place in the apparent absence of ascorbic acid.

The study of the rôle of a particular substance in the secretory mechanism raises fresh problems. How does the substance reach the place of its required reaction, and what is its subsequent fate? With respect to ascorbic acid both these questions have been investigated, but their discussion is reserved for a more extensive report.

SUMMARY

The interstitial tissue of the stroma of the ciliary processes contains a group of reducing substances, one component of which (vitamin C) has been identified as ascorbic acid. These substances act as a mediating system, facilitating the oxidation-reduction interaction between the stroma cells and the stroma-epithelium barrier.

Deprivation of vitamin C from the diet of guinea pigs leads to a decrease of this substance in the ocular tissues before any constitutional symptoms of scurvy develop. The loss of ascorbic acid from the secretory system in the eye results in a decrease in the oxidation-reduction interaction between stroma and epithelium—in a decrease in the rate of secretion of the intra-ocular fluid.

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DISCUSSION

DR. FRANCIS H. ADLER, Philadelphia, Pa.: I cannot resist the temptation of reminding you that during the past few years the opposing forces of those who have advocated the theory of secretion of the aqueous, and those who have advocated the theory of dialysis, have been meeting in deadly combat. Largely as a result of Dr. Friedenwald's painstaking work, which I believe is epoch-making, even the most ardent champion of the dialysate theory—Duke Elder—has now partially lowered the flag, and admitted that dialysis alone cannot account for the formation of aqueous.

HYPERPHORIA TESTS BASED UPON A NEW PRINCIPLE*

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For the purposes of this communication it is necessary to define binocular fixation, for, so far as I know, this has never been accurately done. In fact, to define it is not generally recognized as constituting a problem. The following definitions seem adequate for the purposes at hand, and I believe will prove of general usefulness. Binocular fixation of two punctate retinal images is the act of maintaining these images upon corresponding retinal points by means of motor response to these images. Corresponding retinal points have been defined by me elsewhere.[1] Binocular fixation of two contours

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