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STIMULATION OF ION TRANSPORT BY ASCORBIC ACID THROUGH INHIBITION OF 3′ : 5′-CYCLIC-AMP PHOSPHODIESTERASE IN THE CORNEAL EPITHELIUM AND OTHER TISSUES*

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SUMMARY

Ascorbic acid stimulates active transport of Cl^- by the isolated intact corneas. The effect is not present in corneas previously stimulated by theophylline, an inhibitor of 3′ : 5′-cyclic-AMP phosphodiesterase (EC 3.1.4.17), and vice versa, theophylline has no action after stimulation with ascorbic acid. This indicated inhibition of 3′ : 5′-cyclic-AMP phosphodiesterase by ascorbic acid. Assay of phosphodiesterase using ^3H-labeled cyclid AMP of frog and rabbit corneal epithelial homogenates showed an inhibitory effect of ascorbic acid. Concentration of 5 mM produced 16 % inhibition with 20 mM producing 46 %. This compares with 58 % inhibition by theophylline at 5 mM. Phosphodiesterase activity is mostly soluble in frog corneal epithelium but in rabbit 45 % is particulate. Soluble and particulate fractions are inhibited by ascorbate, but in rabbits greater inhibition (50 %) was observed in the particulate fraction than in the soluble fraction. Other tissues showed inhibition also: frog retina 12 %, rat brain (caudate nucleus) 48 %, rabbit brain 14 %, rabbit liver 16 %. It is concluded that ascorbate produces an increase in cyclic AMP content of corneal epithelium and other tissues by inhibition of 3′ : 5′-cyclic-AMP phosphodiesterase. This action may be one of the main functions of the high ascorbic acid content of ocular tissues and explain some of the effects of high dosis of ascorbate in other systems.

INTRODUCTION

Ascorbic acid is concentrated in the aqueous humor of the eye of many species by a mechanism of active transport from blood to eye which resides in the epithelium lining the ciliary body [1, 2]. Despite a large number of reports the reason why this high ascorbic acid concentration should exist in the eye remains unclear. In other

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tissues there are indications for a role of ascorbic acid in specific enzyme reactions [3].

In this paper an inhibitory action of ascorbic acid on the 3′ : 5′-cyclic-AMP phosphodiesterase (EC 3.1.4.17) activity of subcellular homogenates of corneal epithelium and other tissues is presented. This investigation was prompted by the observation [4] that ascorbic acid in very low concentration (10^{-5} M) stimulated the active transport of Cl^- of the toad corneal epithelium. Our investigations, presented in this paper, showed that in intact in vitro corneas there was an antogonism between the known response to theophylline [5] and the response to ascorbic acid.

Since theophylline is known to inhibit 3′ : 5′-cyclic-AMP phosphodiesterase in subcellular fractions of most tissues, our attention was directed towards this enzyme and its possible relationship to ascorbic acid. As shown in the results presented here, there is an inhibition of this enzyme by ascorbic acid and the indication is that probably an increase in the cyclic AMP content of the corneal epithelium is produced by ascorbic acid. Other tissues, ocular and nonocular, were examined also and inhibition was found; however, the extent of inhibition varied greatly between tissues.

MATERIALS AND METHODS

Electrical measurement of ion transport

Corneas were dissected out from pithed specimens of *Rana catesbeiana* placed in Ringer solution and mounted as a membrane between lucite hemichambers. The two surfaces were bathed with Ringer's solution and spontaneous electrical potential difference and the short-circuit current were followed continuously with an automatic voltage clamp system. The general methods utilized were the same as described by Zadunaisky [6], Zadunaisky and Lande [7], and Zadunaisky et al. [8] Ascorbic acid, theophylline and epinephrine were prepared immediately before use as stock solutions in Ringer's solution, pH adjusted to 7.4. From these stock solutions, volumes between 5 and 100 μl were added to the fluid bathing the cornea in order to achieve final concentrations between 10^{-3} and 10^{-5} M.

Preparation of tissue homogenates

Frog corneal epithelium. Bull frogs (*Rana catesbeiana*) were pithed and corneal buttons immediately removed by use of a trephine. The epithelium was removed with two different methods. One consisted in scraping the epithelium with a scalpel and placing it in a glass tissue homogenizer containing cold Tris buffer (0.1 M Tris · HCl, pH 7.5, containing 3 mM $MgSO_4$). The second method consisted in placing the whole cornea in the glass tissue homogenizer with ground glass surface containing Tris buffer and homogenizing off the epithelium by use of a ground glass pestle. The remaining corneal stroma was removed by means of forceps and the epithelial cells homogenized further. All homogenizations were done at 4 °C. The homogenate was diluted to give a final concentration of one epithelium from one cornea per ml of Tris buffer and was used without further manipulation for assay of phosphodiesterase activity. Fresh preparations of frog corneal epithelium were made each day.

Rabbit corneal epithelium. Frozen mature albino rabbit corneas were obtained from Pel-Freeze, Corp., Ark. The corneas were trimmed of scleral tissue and the epithelial layer was removed and homogenized as above. The final concentration of the homogenate was 1.5 corneas per ml of Tris buffer.

Frog retina. Bull frogs were dark adapted for 2 h before pithing, the eyes were immediately enucleated and hemisected and the retina removed from the pigment epithelium by use of forceps. All the above procedures were done in dim light. The retinas were placed in cold Tris buffer and homogenized at 4 °C in a glass tissue homogenizer with teflon pestle. The homogenate was diluted to give a final concentration of one retina per 2 ml of buffer. Fresh homogenates were prepared each day.

Rabbit heart, liver and whole brain. These tissues were removed from mature albino rabbits immediately after asphyxiation by CO_2 and placed on ice. Heart was carefully trimmed of membranes, vessels and fat, cut into small pieces and washed five times with 20 vol. of ice-cold water, before homogenization; liver and whole brain were homogenized as removed. Homogenization was done using the semi-micro attachment to the Waring blender at 4 °C. Each tissue was blended for 3 min at maximum speed. The homogenate was diluted to give a final concentration of 1 g of tissue per 10 ml of Tris buffer.

Preparation of reagents

All reagents used were analytical grade. Theophylline, cyclic 3′ : 5′-AMP and 5′-AMP were from Sigma Chemical Co. [^{3}H]Adenosine-3′ : 5′-cyclic monophosphate acid was from New England Nuclear, No. ET-275, (specific activity 24.1 Ci/mmol). Ascorbic acid was from Fischer Chemical Co. $Ba(OH)_2 \cdot 8H_2O$ was from Baker Chemical Company. Substrate was prepared fresh on day of assay. 40 μl of stock cyclic [^{3}H]AMP solution, 0.1 ml of 5 mM cyclic AMP and 0.9 ml Tris buffer. If 5′-AMP was required in the assay mixture 0.1 ml of 0.1 mM 5′-AMP was added to the substrate and only 0.8 ml of buffer. The amount of labeled cyclic AMP added was sufficient to give approximately 10 000 cpm when the assay procedure was run in absence of enzyme (tissue homogenate).

Assay of 3′ : 5′-cyclic-AMP phosphodiesterase activity

Phosphodiesterase activity was assayed according to the radioactive method of Poch [9]. Unless otherwise stated, each reaction mixture consisted of 0.4 ml of tissue homogenate, 0.1 ml of inhibitor if any and an amount of Tris buffer (0.1 M Tris · HCl, pH 7.5, containing 3 mM $MgSO_4$) to give a final volume of 1.0 ml and to start reaction 0.1 ml of labeled substrate. The final substrate concentration was $0.5 \cdot 10^{-4}$ M. In all assays except those for cornea 0.1 ml of 0.1 M 5′-AMP was added to the substrate to give a final volume of 1 ml in the reaction mixture. Assays on corneal homogenates showed no difference between those run in the presence or absence of added 5′-AMP and therefore corneal assays were routinely run without the addition of 5′-AMP.

RESULTS

Activation of ion transport by ascorbic acid and antagonism to theophylline

The addition of ascorbic acid produced an increase in the short-circuit current of the isolated corneas tested. Conditions were such that this current practically entirely represented active Cl^- transport [6].

Fig. 1 shows this response to 1 mM ascorbic acid which is in the range of the

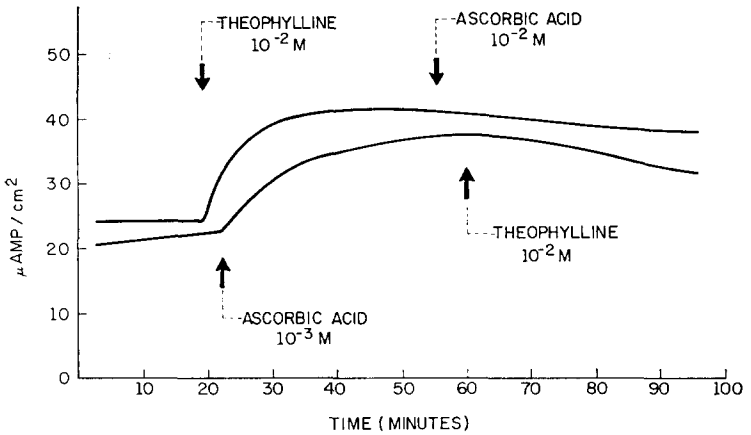

Fig. 1. Effect of ascorbic acid on the Cl^- short-circuit current of 2 isolated frog corneas. Note that theophylline has no effect when added at plateau of ascorbic acid stimulation and vice versa, ascorbic acid has no further effect when added at the plateau of the stimulation by theophylline.

normal concentration in the aqueous humor. Lower concentrations such as 10^{-4} and 10^{-5} molar produced smaller, but detectable and proportional increases in the short-circuit current. In the same Fig. 1 it can be observed that addition of theophylline at the plateau of the increase produced by ascorbic acid had no effect. On the contrary, theophylline applied on a fresh preparation produces the already described increase in current. On the other hand, if now ascorbic acid is added on at the plateau of the response to theophylline, there is no increase in the current.

Phosphodiesterase activity of frog corneal epithelial homogenates

The activity of frog cornea epithelium versus reaction is essentially linear up to 90 min of incubation time. Because of the difficulty inherent in obtaining large quantities of frog epithelium, 90-min incubations were routinely used in order to obtain significantly more cpm.

Ascorbic acid inhibition of phosphodiesterase of frog corneal epithelium

At a final concentration of 10 mM in the reaction mixture ascorbic acid caused a 37 % inhibition of phosphodiesterase activity of frog corneal epithelium. The average of 17 determinations each done on a different day with a different tissue preparation is presented in Fig. 2A. There is a highly statistically significant difference ($P < 0.01$) between the control activities and the lower activities found in the presence of ascorbic acid.

For comparison Fig. 2A also gives the inhibition of phosphodiesterase by theophylline. Theophylline at a final concentration of 5 mM caused an average of 58 % inhibition.

Shorter incubation times (30 min) were also assayed for ascorbate inhibition and showed essentially the same results as those at 90 min.

In order to rule out the possibility that the phosphodiesterase inhibition in the presence of ascorbic acid was due to a change in the pH of the reaction medium during the incubation period, the pH of the reaction mixture was determined at the

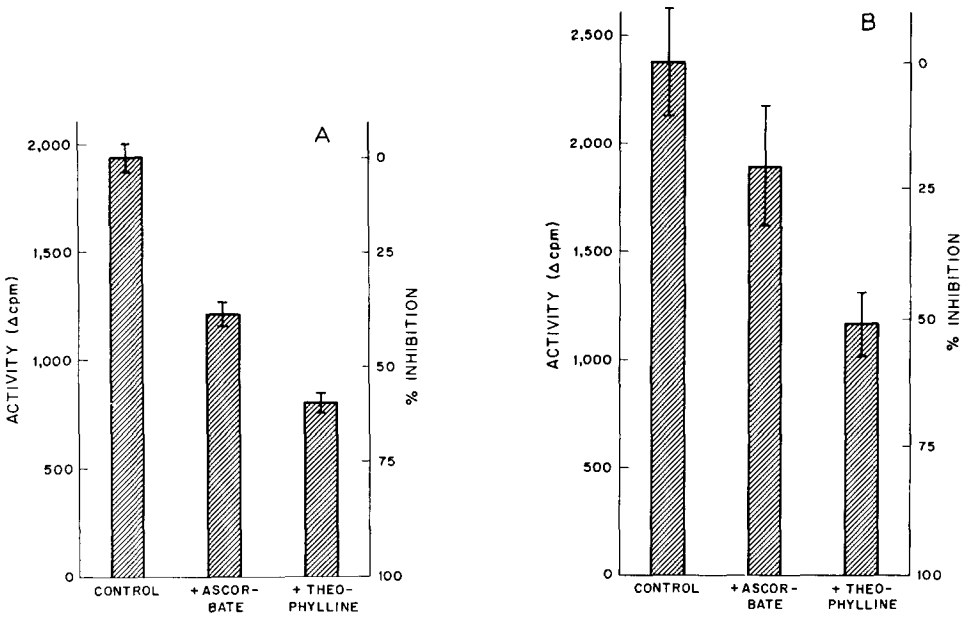

Fig. 2. Inhibition of phosphodiesterase activity of frog (A) and rabbit (B) corneal epithelium by ascorbic acid. A. Average of 17 determinations each done with freshly prepared corneal epithelium on different days. The theophylline results are the average of 5 determinations. The difference between control and inhibited is statistically significant ($P < 0.01$). B. Average of 5 determinations. The theophylline result is the average of 2 determinations. The difference between control and inhibited data is statistically significant ($P < 0.01$).

start and end of the incubation period. There was essentially no change in pH.

The inhibitory response of frog corneal epithelial phosphodiesterase activity to varying concentrations of ascorbic acid is shown in Fig. 3. At 1 mM concentration ascorbic acid shows no inhibitory action. At higher concentrations of 5, 10, and 20 mM the percent inhibition is 16, 30, 46 % respectively. Kakiuchi et al. [12] have reported that the phosphodiesterase activity found in the soluble fraction of rat brain cortices homogenate is Ca^{2+} dependent. It is unlikely the inhibitory effect of ascorbic acid is due to an effect of ascorbic acid on Ca^{2+}. The addition of 10^{-5} M Ca^{2+} (added as calcium gluconate) had no effect on the uninhibited phosphodiesterase activity of frog corneal epithelium and no effect on the extent of ascorbate inhibition. The addition of a higher concentration of Ca^{2+} (10^{-3} M) in one experiment caused a 33 % inhibition of the phosphodiesterase activity; however, this concentration of Ca^{2+} still showed no effect on the percent of ascorbate inhibition.

Effect of ascorbic acid on phosphodiesterase activity of rabbit corneal epithelium

Similar to its action on frog cornea, ascorbic acid inhibits the phosphodiesterase activity of rabbit corneal epithelium. 10 mM ascorbate caused a 20 % inhibition. Fig. 2B represents the average of 5 determinations with an inhibitory range of 5–47 %. Theophylline at a final concentration of 5 mM caused an average of 50 % inhibition.

The inhibitory effect of ascorbate on rabbit cornea appears to be lower than

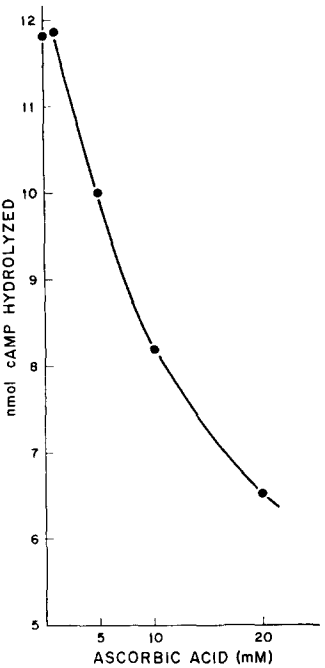

Fig. 3. Ascorbate inhibition of frog corneal phosphodiesterase activity as a function of ascorbate concentration. The standard assay reaction mixture was used with additions of stock ascorbic acid solution replacing equivalent amounts of buffer. Reaction time was 90 min.

TABLE I

INHIBITORY EFFECT OF ASCORBIC ACID ON PHOSPHODIESTERASE ACTIVITY OF SEVERAL TISSUES

Tissues	Additions	nmol hydrolyzed	% inhibition
Toad cornea	None	28.7	—
Toad cornea	10 mM ascorbate	23.1	20
Frog retina	None	43.0	—
Frog retina*	10 mM ascorbate	38.0	12
Rat caudate nucleus**	None	39.8	—
Rat caudate nucleus	10 mM ascorbate	20.6	48
Rabbit brain	None	41.5	—
Rabbit brain	10 mM ascorbate	35.7	14
Rabbit liver	None	40.5	—
Rabbit liver	10 mM ascorbate	34.0	16
Rabbit heart	None	41.7	—
Rabbit heart	10 mM ascorbate	40.4	3

* With the use of the method of Thompson and Appleman [14] inhibitions up to 90% of frog retinal homogenates by 10 mM ascorbate were found. Also in rod outer segments, ascorbic acid proved to be a potent inhibitor of phosphodiesterase (Chadder, J., personal communication).

** Rat caudate nucleus was a generous gift of G. Petzold of P. Greengard's Laboratory. In another experiment using a different preparation of rat caudate nucleus, G. Petzold observed a 5.5% inhibition of phosphodiesterase activity by 3 mM ascorbate and only 16.5% inhibition by 10 mM ascorbate.

that observed with frog cornea, 20 vs 37 % respectively. However, this difference may reflect the fact that frozen corneas were used for rabbit samples versus the use of fresh frog corneas. In one experiment, phosphodiesterase activities with and without addition of ascorbate were measured on aliquots of fresh and after freezing frog corneal epithelium and lower values were found.

Effect of ascorbic acid on phosphodiesterase activity of other species and tissues

As might be expected, the phosphodiesterase activity of corneal epithelium from toad (species *Bufus marinus*) exhibited an inhibitory sensitivity comparable with that of frog and rabbit. This result as well as the ascorbate sensitivity of other tissues is given in Table I. The inhibition by 10 mM ascorbate of phosphodiesterase activity of the frog retina (12 %) is considerably less than that observed with frog cornea (37 %). However, in recent preliminary experiments using the method of Thompson and Appleman [14] inhibitions up to 90 % were found in frog retina homogenates. The effect of ascorbic acid on the much studied phosphodiesterase of mammalian brain was of considerable interest. The phosphodiesterase of whole rabbit brain was 14 % inhibited by 10 mM ascorbate. However, rat caudate nucleus which has considerably more phosphodiesterase activity than whole brain exhibited in one experiment a high sensitivity toward ascorbate, showing an inhibition of 48 % by 10 mM ascorbate. Rabbit liver phosphodiesterase activity was 16 % ascorbate inhibited. Rabbit heart phosphodiesterase activity showed very little ascorbate sensitivity being only 3 % inhibited by 10 mM ascorbate.

Subcellular distribution of frog and rabbit corneal epithelial phosphodiesterase

For fractionation frog and rabbit corneal epithelium were prepared as described under Materials and Methods. A portion of the whole homogenate was sedimented at 100 000 $\times g$ for 60 min. The supernatant solution represents the soluble fraction. The precipitate was homogenized in 0.1 M Tris · HCl buffer, pH 7.5, containing 3 mM $MgSO_4$ and represents the particulate fraction.

TABLE II

SUBCELLULAR DISTRIBUTION OF PHOSPHODIESTERASE ACTIVITY OF FROG AND RABBIT CORNEAL EPITHELIUM

Freshly prepared corneal epithelial homogenate was centrifuged at 100 000 $\times g$ for 60 min. The supernatant fluid is the soluble fraction; the precipitate, resuspended to the original volume, the particulate fraction. For the enzyme assay of each fraction, 0.4 ml (as described under Materials and Methods) of sample was used per reaction mixture. The relative phosphodiesterase activities of the soluble and particulate fractions are expressed as a percentage of whole homogenate activity which is set at 100 %. The percentage inhibition by 10 mM ascorbate for each fraction is the percentage relative to the uninhibited sample.

Fraction	% phosphodiesterase activity		% inhibition by 10 mM ascorbate	
	Frog	Rabbit	Frog	Rabbit
Whole homogenate	100	100	42	20
Soluble fraction	108	66	36	19
Particulate fraction	9	25	61	53

The phosphodiesterase activity of frog cornea is found almost entirely in the soluble fraction (Table II). Less than 9 % of the activity relative to the whole homogenate was found in the particulate fraction. These results are an average of 5 determinations. Other workers [15–17] using different tissues and somewhat different conditions found most of the phosphodiesterase activity in the particulate fraction. Our fractionations were repeated using isotonic sucrose medium similar to that used by Butcher and Sutherland [15] and still we found nearly all phosphodiesterase activity in the supernatant fractions. Drummond and Penott [17] using brain recovered all activity in the $100\,000 \times g$ supernatant fraction. Furthermore, unlike the Cheung and Salganicoff [18] finding in rat brain, the addition of 2 % cholate or 0.5T Triton X-100 to the whole homogenate had no effect on the phosphodiesterase activity giving additional support to the observation that phosphodiesterase of frog cornea is found in the soluble fraction.

Cheung [19] has reported the presence of a heat-stable protein activator of phosphodiesterase in the soluble fraction. Incubation of precipitate fraction with boiled supernatant fraction had no effect on phosphodiesterase activity of the particles.

In contrast to frog, rabbit cornea showed considerable amounts of phosphodiesterase activity in the particulate fraction. The results presented also in Table II show a distribution of 25 % residing in the particulate fraction.

Table II also gives the percent inhibition of phosphodiesterase activity by 10 mM ascorbate in the various fractions. The soluble fraction exhibited essentially the same inhibitory response as the whole homogenate (20 %). The particulate fraction however exhibited a much higher ascorbate sensitivity being 53 % inhibited by the 10 mM ascorbate. Frog cornea showed a similar effect.

DISCUSSION

The interaction of ascorbic acid and theophylline on the Cl^- transport of the intact isolated frog cornea indicated that ascorbic acid could have some relationship to the metabolism of cyclic AMP. The absence of response to theophylline after ascorbic acid is clear cut and the opposite phenomenon is also found; that is, no response to ascorbate after theophylline. The time course of the response, at least in the frog cornea is very similar between these two agents. The possibility exists then that ascorbic acid could act by producing an increase of cyclic AMP in the corneal epithelium. In fact, epinephrine produces a dramatic increase in the Cl^- transport of both frog and rabbit cornea [5–8, 10] due to an increase in cyclic AMP content of the tissues. Theophylline increases the transport rate presumably by inhibition of phosphodiesterase also in this tissue.

Further evidence that the stimulation of active ion transport across the cornea by ascorbic acid results from an increase in cyclic AMP content of corneal epithelium caused by ascorbate inhibition of 3′ : 5′-cyclic-AMP phosphodiesterase comes from the assays of phosphodiesterase activity of corneal epithelial homogenates in the presence of ascorbic acid. Ascorbic acid inhibits phosphodiesterase activity. At 5 mM concentrations the inhibition of frog corneal epithelium was 16 % and the inhibition increased with increasing ascorbate concentrations reaching 46 % in the presence of 20 mM ascorbate. Ascorbic acid is not as effective an inhibitor of phosphodiesterase

as the better known theophylline which under our assay conditions caused a 58 % reduction in activity at 5 mM concentrations.

The inhibitory effect of ascorbic acid on phosphodiesterase activity is not limited to amphibian species or to corneal tissue. Rabbit cornea as well as brain and liver and rat caudate nucleus showed significant inhibitory effects by ascorbate. The extent of inhibition varied quite widely. These variations may reflect differences in the phosphodiesterase enzymes of the individual tissues. Two types of cyclic AMP phosphodiesterase activities have been reported in rat brain and other tissues [14, 20, 21] and it appears that one type is localized in the particulate and the other in the soluble fraction [13]. The activity of each may be regulated by different factors and only a portion of the total phosphodiesterase activity therefore may be highly sensitive to ascorbate inhibition. This could explain the variation in ascorbate inhibition of phosphodiesterase activity in frog corneal homogenates by 5 mM ascorbate as compared with a maximal effect by 1 mM ascorbate on ion transport in whole corneas. Another possible explanation for the relatively high ascorbate concentrations required and the tissue variation observed is that ascorbate, i.e. the reduced form may not be the active inhibitory species [22, 23]. Also disruption of the membraneous structure by homogenization may disrupt a favorable spatial arrangement between the ascorbate-oxidizing enzymes and phosphodiesterase.

There is other evidence, however, suggesting different phosphodiesterase enzymes in cornea, one of which is highly sensitive to inhibition by ascorbate. In frog corneal preparations where a low activity (less than 9 % of the total activity) was recovered in the particulate fraction, this phosphodiesterase activity was 61 % inhibited by ascorbate as contrasted with only a 36 % inhibitory effect of ascorbate on the bulk of the enzyme activity found in the soluble fraction. This finding is more evident in experiments with rabbit cornea where 25 % of the phosphodiesterase activity remains bound to the particulate fraction. The particulate enzyme is 58 % inhibited by 10 mM ascorbate while the phosphodiesterase enzyme activity in the soluble fraction is only 20 % inhibited by ascorbate.

The finding that freezing causes a loss in sensitivity to ascorbate inhibition may indicate either that only a portion of the phosphodiesterase activity is sensitive to ascorbate and this portion is more easily destroyed or that freezing modifies the ascorbate interacting site on the enzyme.

It is tempting to speculate that phosphodiesterase is an allosteric enzyme and that ascorbic acid is one of the regulatory agents acting at specific allosteric sites on the enzyme. Appleman [13] has postulated that the low-K_m enzyme is a single enzyme with negatively cooperative allosteric sites.

Finally, the possibility exists that several of the functions of ascorbic acid, including those in the eye, where it is found at such a high concentration, could be mediated by cyclic AMP, in view of the inhibition of its phosphodiesterase shown here.

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