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Ascorbic Acid: Cofactor in Rabbit Olfactory Preparations

Abstract. *A stimulant-induced decrease in absorbance at 267 nanometers in preparations from rabbit olfactory epithelium requires ascorbic acid as a cofactor. Vitamin C is bound to proteins in the olfactory mucosa in vivo. When the stimulant 3,7-dimethyl-6-octen-3-ol interacts with olfactory proteins, it triggers a change in protein conformation which renders ascorbic acid available for oxidation. The specific decrease in absorbance at 267 nanometers is produced when the "freed" ascorbic acid is oxidized.*

Ascorbic acid was identified as a cofactor required for the stimulant-induced changes observed in preparations from rabbit olfactory epithelium. The stimulant-induced changes were monitored with ultraviolet difference-spectroscopy techniques (*1*). Absorbance changes induced at 267 nm were described (*2*); linalool and linalyl isobutyrate were the stimulants. The decrease in specific absorbance at 267 nm was also induced by 3,7-dimethyl-6-octen-3-ol (DMO).

The olfactory preparations contained a cofactor of relatively low molecular weight; it was required for the stimulant-induced decreases in absorbance at 267 nm. Activity was lost when the cofactor was separated from protein fractions of high molecular weight. Several attempts (with dialysis and gel filtration) to isolate the active cofactor and add it to the protein fractions failed because the cofactor was inactivated during isolation. The cofactor was identified as ascorbic acid.

When freshly prepared ascorbic acid solutions were mixed with the olfactory preparations, the stimulant-induced activity at 267 nm was increased. Addition of ascorbic acid also restored the activity in preparations which were depleted of cofactor by dialysis or gel filtration. In the representative experiment ascorbic acid was added to the olfactory preparation to a final concentration of $5.0 \times 10^{-5}M$; the absorbance change induced by DMO was more than doubled (Fig. 1). The change after 30 minutes was 0.041 absorbance units compared to 0.100 units for the duplicate reaction mixture containing $5.0 \times 10^{-5}M$ additional ascorbic acid (Fig. 1a). After the reaction period the absorbance spectra of the reaction mixtures from 240 to 310 nm were determined approximately 40 minutes

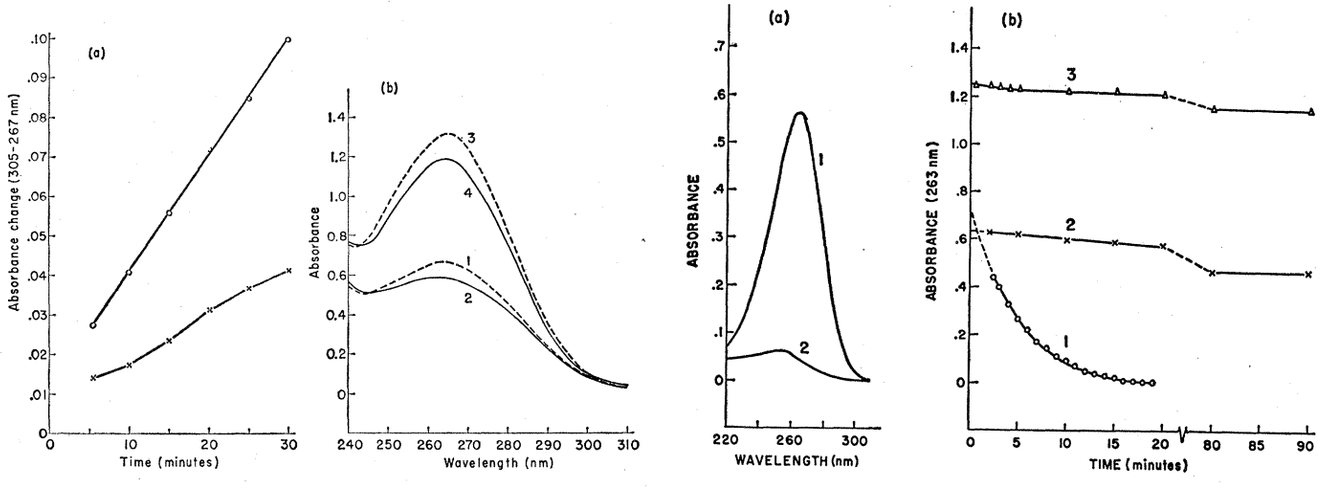

Fig. 1 (left). Effect of ascorbic acid on stimulant-induced absorbance change in olfactory preparations (a) and the absorbance spectra of the olfactory preparations (b). The reaction mixtures each contained 0.70 mg of protein in a final volume of 3 ml. Where added, the final concentration of ascorbate was $5 \times 10^{-5}M$, and final concentration of the stimulant (3,7-dimethyl-6-octen-3-ol) was $3.0 \times 10^{-3}M$. The *p*H was 7.0 and controlled by $0.07M$ phosphate buffer. For the lower curve of part (a) the reaction mixtures combined olfactory preparation, stimulant, and phosphate buffer. The only change for the upper curve was the addition of ascorbate prior to addition of the stimulant. For the absorbance spectra in part (b) the following components were compared after a reaction period of approximately 40 minutes: curve 1, olfactory preparation and buffer are plotted against buffer; curve 2, olfactory preparation, buffer, and DMO are plotted against buffer and DMO; curve 3, olfactory preparation, buffer, and ascorbate are plotted against buffer; curve 4, olfactory preparation, buffer, ascorbate, and DMO are plotted against buffer and DMO. Fig. 2 (right). Oxidation of ascorbate and the stabilizing of proteins. (a) The spectrum of $5 \times 10^{-5}M$ ascorbate in $0.09M$ phosphate buffer, *p*H 7.0, was determined approximately 1 minute after preparation; curve 1 of part (a); curve 2 is the same solution after having been shaken with air for 3 minutes. (b) The reaction mixture for curve 1 contained $5 \times 10^{-5}M$ ascorbate in phosphate buffer. For curve 2 the reaction mixture also contained 0.3 mg of bovine serum albumin per milliliter, and for curve 3 it contained olfactory preparation (0.43 mg of protein per milliliter) in addition to the ascorbate. The absorbance was followed on the Cary 14 spectrometer.

after stimulant addition (Fig. 1b). The intensity of the absorbance maxima was increased considerably but the position of the maxima was unchanged by addition of the highly absorbing ascorbic acid. Also, the position of the greatest change in the difference spectra was still at 267 nm distinct from the 263 nm absorbance maximum.

Ascorbic acid concentration in our rabbit olfactory preparations (*3*) ranged from 11.7 to 80.0 μg of ascorbic acid per milligram of protein. The standard deviation was ± 1 μg of ascorbic acid per milligram of protein at a concentration of 24 μg/mg. The highest amounts of ascorbic acid and also the greatest activities were found when the animals' diet was supplemented with ascorbic acid for several days prior to their being killed for samples.

Free ascorbate is not stable in aqueous media at *p*H 7.0, and thus requires a stabilizer (*4*). However, the location of the absorbance peaks can be determined even though the intensity of the peak changes quite rapidly. The absorbance spectrum of $5.0 \times 10^{-5} M$ ascorbate in 0.09*M* phosphate buffer at *p*H 7.0 was determined approximately 1 minute after its preparation (Fig. 2a). After shaking the ascorbate solution for 3 minutes to oxidize the ascorbate to dehydroascorbate, the absorbance at 263 nm was greatly reduced. When dehydroascorbate was added to the olfactory preparations there was no stimulation of the activity at 267 nm; this explained why earlier attempts to isolate the active cofactor from the olfactory preparations failed. The rate of oxidation of "free" ascorbate under these conditions was determined (Fig. 2b). After 15 minutes at 25°C the absorbance at 263 nm had decreased by 98 percent of its initial value. The absorbance at time zero was determined by extrapolation. The absorptivity calculated from the absorbance at time zero agreed with the value reported by Szepesy (*4*).

A portion of the ascorbic acid in animal tissues is bound to proteins (*5*). In the experiments of Sumerwell and Sealock, about 10 percent of the ascorbic acid present in rat liver homogenates was precipitated by 95 percent ethanol. A portion of the ascorbic acid is bound to proteins in vivo (*5*).

Even at *p*H 7.0, the ascorbate was protected against rapid oxidation if proteins were included in the reaction mixture. Either bovine serum albumin or the olfactory preparation was effective in stabilizing the ascorbate (Fig. 2b). After 15 minutes the absorbance at 263 nm decreased by only about 3 percent in the solution containing both ascorbate and olfactory preparation; after 90 minutes the absorbance at 263 nm was still only decreased by 8 percent of the initial value. Bovine serum albumin effectively protected against ascorbate oxidation (Fig. 2b). However, the mixture of ascorbate and bovine serum albumin did not support the stimulant-induced activity at 267 nm.

The rate of oxidation of "free" ascorbate was much faster than the rate of the decrease of stimulant-induced absorbance (Fig. 2b). Five minutes after mixing, the absorbance of the ascorbate solution had decreased from 0.73 to 0.27 units; the rate of this reaction is approximately 20 times that of the decrease in stimulant-induced absorbance. Therefore, the oxidation of "free" ascorbate was not the rate-limiting step of the stimulant-induced activity. Also, since difference spectroscopy was used to monitor the stimulant-induced activity, any changes not associated with the stimulant interactions would take place in both chambers of the spectrophotometer and would therefore cancel one another.

The results from four different experimental approaches all indicate that the change in stimulant-induced absorbance at 267 nm involves interactions of stimulant and protein. First, activity was destroyed when the preparations were heated to 68°C for denaturation of the proteins (*2*). Second, the stimulant-induced activity was maximum between *p*H 6.5 and 7.5. Third, activity of the olfactory preparations was destroyed in the presence of 8*M* urea which alters the secondary and tertiary structure of proteins in solution (*3*). Fourth, it has been proposed (*6*) that the depth of the trough at 233 nm in optical rotatory dispersion spectra of proteins is related to the α-helix content of the proteins (*7*). In our experiments the stimulant-induced activity was accompanied by a change in the α-helix content of the olfactory preparations as measured by optical rotatory dispersion. The depth of the trough at 233 nm was decreased by about 9 percent when the olfactory preparation was stimulated with 9.0 m*M* DMO.

The scraping method for obtaining the olfactory preparations from rabbits (*2*) was replaced by an improved technique which involved soaking the turbinate and posterior portion of the nasal septum in isotonic phosphate buffer (*p*H 7.0) prior to centrifugation. The amount of active preparation per rabbit was increased by more than tenfold and the specific activity (absorbance change per milligram of protein) was more than doubled.

Ascorbic acid is a dialyzable cofactor in the olfactory preparations; it is required for the stimulant-induced activity monitored by change in absorbance at 267 nm. Ascorbic acid in the olfactory preparations is bound to proteins; in this bound form it is stabilized against oxidation to dehydroascorbic acid. Introduction of the stimulant (DMO in our experiments) triggers a change in protein conformation which renders the ascorbic acid available for oxidation. The decrease in absorbance monitored at 267 nm is due to oxidation of the "freed" ascorbic acid. Electron microscopy studies support the hypothesis that components of the olfactory preparations are associated with the surface of the biological sensors in vivo. The stimulant-induced change in conformation might therefore represent the transduction step in olfaction which alters permeability of the cell membrane and thereby initiates production of the electrical impulse which is transmitted to the brain for interpretation.

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