PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 164, 534–536 (1980)

# Ascorbic Acid Inhibition of Cyclic Nucleotide Phosphodiesterase Activity (40911)[1]

DANIEL MALAMUD AND YALE KROLL

*Department of Biochemistry, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

*Abstract.* Low levels of ascorbic acid reversibly inhibit mouse brain and beef heart cyclic nucleotide phosphodiesterase activity *in vitro*. Inhibition is noncompetitive with a $K_i \simeq 10^{-4}$ *M*. Similar inhibition occurs with D-ascorbate but not with dehydroascorbate or metabisulfite. The kinetics of inhibition by ascorbic acid were similar for "high" and "low" $K_m$ cAMP phosphodiesterase and cGMP phosphodiesterase activity.

There have been conflicting reports in the literature on the ability of ascorbic acid or its derivatives to inhibit cyclic nucleotide phosphodiesterase activities obtained from various tissues. Tilsdale (1) reported that both ascorbic acid and dehydroascorbic acid inhibited cAMP phosphodiesterase activity from Walker carcinoma cells. The latter inhibition was noncompetitive while the former appeared competitive with $K_i$ values $\simeq 10^{-4}$ *M*. Stimulation of cGMP levels by dehydroascorbate in guinea pig spleen (2) or by ascorbate and dehydroascorbate in human lymphocytes (3) occurs without cGMP phosphodiesterase inhibition. In contrast, stimulation of $Cl^-$ transport in corneal epithelium by ascorbic acid was associated with an inhibition of cAMP phosphodiesterase activity (4).

*Materials and methods.* Whole mouse brains, or other tissues as indicated, obtained from male CD-1 mice were homogenized in 0.25 *M* sucrose, 50 m*M* Tris, and 10 m*M* $MgCl_2$, pH 7.5. All chemicals were reagent grade. Ascorbic acid (Fisher) was dissolved in buffer and the pH adjusted to 7.5. Aliquots of the homogenate were centrifuged at 20,000*g* for 20 min to give a soluble fraction. cAMP and cGMP phosphodiesterase activities were determined by monitoring the appearance of [$^3$H]nucleoside formed from [$^3$H]cAMP or [$^3$H]cGMP as described by Thompson and Appleman (5) using both zero time and no enzyme blanks. Reactions were incubated at 30° for 10 min. Under these conditions enzyme activity was first order.

*Results.* At high substrate concentrations ($10^{-5}$ *M* cAMP), ascorbic acid inhibited brain cAMP phosphodiesterase in a dose-dependent fashion. In contrast, there was no inhibition of cAMP phosphodiesterase activity obtained from several other mouse tissues (Fig. 1). Inhibition of phosphodiesterase activity was not seen with other antioxidants such as dithiothreitol (0.1–1 m*M*, not shown) or sodium metabisulfite (Fig. 2).

Inhibition was not stereospecific but did require the reduced form of ascorbic acid, since dehydroascorbate actually stimulated enzyme activity (Fig. 2). The inhibition of brain phosphodiesterase activity by ascorbate was fully reversible. When enzyme preparations were incubated with 1 m*M* ascorbate, the inhibition could be reversed by overnight dialysis or Amicon filtration. The dialyzed enzyme preparation was still susceptible to inhibition by added ascorbate. Inhibition of brain phosphodiesterase by ascorbate was independent of the pH of the reaction mixture between pH's 7 and 9.

Kinetic studies of mouse brain phosphodiesterase activity revealed two $K_m$'s for cAMP and one $K_m$ for the cGMP (Table I). To estimate the effect of ascorbate on each of the activities, substrate concentrations near the apparent $K_m$'s were selected for subsequent assays. As shown in Fig. 3, ascorbate was equally effective as an inhibitor of mouse brain cAMP phosphodiesterase at high and low substrate concentrations. A similar dose–response relationship was obtained for cGMP ($10^{-5}$ *M*) hydrolysis.

[1] This research was supported by N.I.H. Grant GM-21700.



0037-9727/80/080534-03$01.00/0

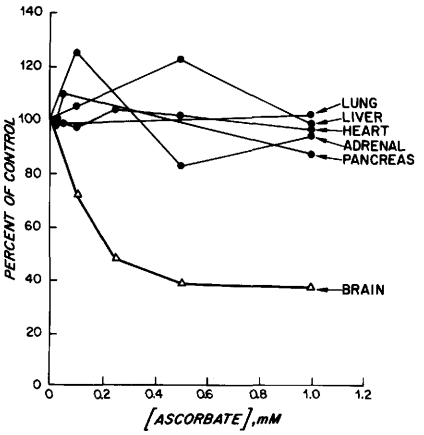


FIG. 1. Inhibition of cAMP phosphodiesterase activity by ascorbate. Aliquots of the 20,000*g* supernatant (5–50 μg protein) were incubated in a reaction mixture containing 50 m*M* Tris–HCl, pH 7.5, 1 m*M* $MgCl_2$, [$^3$H]cAMP ($10^{-5}$ *M*, 20,000 cpm) and ascorbate at the indicated concentrations. Reactions were incubated at 30° for 10 min and terminated in a boiling water bath for 2 min. [$^3$H]cAMP was converted to [$^3$H]adenosine and assayed as described by Thompson and Appleman (5). Enzyme activity is expressed relative to the control (no drug). Each point is the average for triplicate determinations.

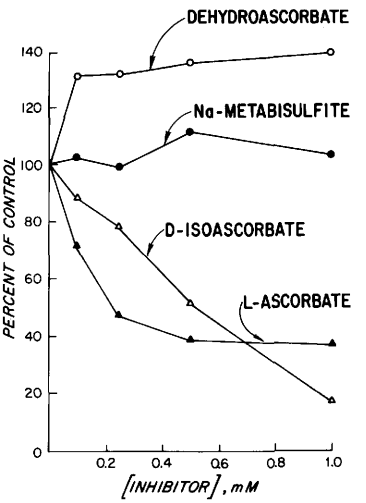


FIG. 2. Inhibition of mouse brain phosphodiesterase by L-ascorbate and related compounds. Aliquots of the 20,000*g* supernatant from mouse brain were assayed as described in Fig. 1. PDE activity relative to the control (no drug) is shown as a function of drug concentration.

Further kinetic studies were carried out with the supernatant preparations from mouse brain to determine the type of enzyme inhibition. For both high and low $K_m$ cAMP phosphodiesterase and for cGMP phosphodiesterase, ascorbate was a noncompetitive inhibitor, decreasing the $V_{max}$ with no effect on the apparent $K_m$ (Table I). We have also looked at the effect of ascorbic acid on beef heart phosphodiesterase purified according to the technique of Butcher and Sutherland (6). Ascorbic acid is a noncompetitive inhibitor of this purified enzyme preparation with an $I_{50}$ of 0.1 m*M*.

*Discussion.* Our experiments have shown that physiologic levels of ascorbate inhibit crude cyclic nucleotide phosphodiesterase obtained from mouse brain. The effective concentration of ascorbate was similar to that reported to increase prolyl hydroxylase activity (7) and hydroxyproline synthesis in fibroblasts (8) and is within the range of brain ascorbic acid levels reported for rats and humans (9).

The apparent tissue specificity of ascorbic acid for inhibition of brain cyclic nucle-

TABLE I. KINETIC PARAMETERS OF MOUSE BRAIN PHOSPHODIESTERASE AND ASCORBATE INHIBITION

| Substrate | Inhibitor | $K_m$ (*M*) | $V_{max}$ | $K_i$ (*M*) |
|---|---|---|---|---|
| cAMP | — | $6 \times 10^{-6}$ | 25 | — |
| cAMP | 1 m*M* ascorbate | $4 \times 10^{-6}$ | 5 | $4 \times 10^{-4}$ |
| cAMP | — | $3 \times 10^{-5}$ | 50 | — |
| cAMP | 0.3 m*M* ascorbate | $2 \times 10^{-5}$ | 8 | $2.3 \times 10^{-4}$ |
| cGMP | — | $0.9 \times 10^{-6}$ | 5 | — |
| cGMP | 0.3 m*M* ascorbate | $1.1 \times 10^{-6}$ | 2 | $2.1 \times 10^{-4}$ |

*Note.* Aliquots of mouse brain 20,000*g* soluble fraction containing 4–10 μg protein were incubated with varying concentrations of substrate (cAMP or cGMP, $10^{-7}$–$10^{-5}$ *M*) in the presence or absence of ascorbate. Data were analyzed by Lineweaver–Burk plots revealing two $K_m$'s for cAMP hydrolysis and one $K_m$ for cGMP hydrolysis. Velocity is expressed as nmoles/mg protein/min.

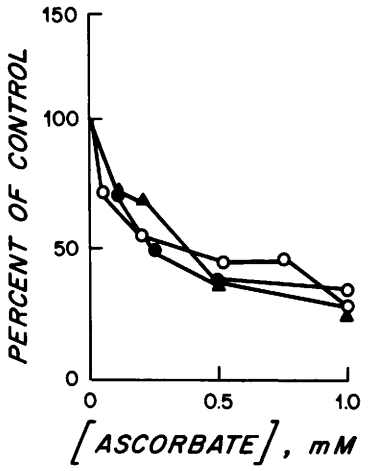


FIG. 3. Inhibition of high and low $K_m$ brain cyclic nucleotide phosphodiesterases. Aliquots of the 20,000*g* supernatant were incubated in reaction mixtures containing $10^{-5}$ *M* cAMP (●), $10^{-5}$ *M* cGMP (○), or $10^{-7}$ *M* cAMP (▲) as substrate. Other details as described in Figs. 1 and 2.

otide phosphodiesterase activities could be the result of interaction with a tissue-specific phosphodiesterase or to an accelerated rate of ascorbic acid catabolism in the other tissues. Preliminary experiments suggest that *in vitro* catabolism of ascorbate may account for some of the observed tissue differences. Thus, while ascorbate produced a dose-dependent inhibition of mouse brain phosphodiesterase activity, incubation of liver supernatant with brain supernatant and ascorbate blocked the inhibition. The observation that ascorbic acid inhibited a purified preparation of beef heart phosphodiesterase suggests that ascorbic acid itself, rather than a metabolite, is the active form of the inhibitor. Dose–response curves for inhibition of mouse brain phosphodiesterase activity by ascorbate leveled off at 60–75% inhibition. Similar results were obtained with two other inhibitors, dipyridamole and papaverine (data not shown). Such incomplete inhibition suggests a form of the enzyme resistant to inhibition by these compounds.

1. Tilsdale, M. J., Biochem. Biophys. Res. Commun. **62**, 877 (1975).
2. Haddox, M. K., Stephenson, J. H., Moser, M. E., and Goldberg, N. D., J Biol. Chem. **253**, 3143 (1978).
3. Atkinson, J. P., Weiss, A., Ito, M., Kelly, J., and Parker, C. W., J. Cyclic Nucl. Res. **5**, 107 (1979).
4. Buck, M. G., and Zadunaisky, J. A., Biochim. Biophys. Acta **389**, 251 (1975).
5. Thompson, W. J., and Appleman, M. M., Biochemistry **10**, 311 (1971).
6. Butcher, R. W., and Sutherland, E. W., J. Biol. Chem. **237**, 1244 (1962).
7. Cardinale, G. J., Stassen, F. L. H., Kuttan, R., and Udenfriend, S., Ann. N.Y. Acad. Sci. USA **258**, 278 (1975).
8. Kao, W. W.-Y., Berg, R. A., and Prockop, D. J., Biochim. Biophys. Acta **411**, 202 (1975).
9. Hornig, N., Ann. N.Y. Acad. Sci. USA **258**, 103 (1975).

Received December 11, 1979. P.S.E.B.M. 1980, Vol. 164.