Role of L-ascorbic acid in the reversal of the monoamine oxidase inhibition by caffeine

A possible role of ascorbic acid in protecting the central nervous system from the toxic effects of endogenous psychotomimetic compounds was discussed by Galzigna, (1970) and by Galzigna & Rizzoli (1970). Also, ascorbic acid deficiency has been indicated as one of the more important symptoms of an altered mental behaviour (Pauling, 1968). We now report the results of a study on the inhibitory effect of caffeine on rat liver mitochondrial monoamine oxidase (MAO) and its reversal by L-ascorbic acid.

The oxidation of catecholamines was followed by measuring the oxygen uptake with a Clark electrode connected via a voltage divider to a Sargent RE recorder. The assay mixture contained in a total volume of 2 ml 170 μmol of Na phosphate buffer pH 7·0, 1·7 μmol of KCN and about 10 mg of mitochondrial protein. The addition of substrates and inhibitors was made by Hamilton syringes and a correction for the volume was introduced after each addition. Mitochondria were obtained from rat liver (Schneider & Hogeboom, 1951) and the protein content measured (Gornall, Bardawill & David, 1949).

The apparent dissociation constants with rat liver MAO for different substrates (all $\times 10^{-3}$M; n = 6) were: tyramine 0·3, 5-HT 1·0, dopamine 1·1, adrenaline 10·0. In each case the proportionality of the reaction velocity and enzyme concentration was verified.

Ascorbic acid (8×10^{-4}M) increased the initial velocity of oxidation with all substrates (1×10^{-4}M), without being itself oxidized, the initial velocity without acid and with acid measured as n At. O_2 min^{-1} mg^{-1} protein being respectively: tyramine 2·6, 3·9; 5-HT 2·0, 3·04; dopamine 2·3, 4·9; adrenaline 0·7, 2·1; noradrenaline 0, 0·5; histamine 0,0. Noradrenaline, which was not utilized under normal conditions, was oxidized when ascorbic acid was present in the incubation mixture. In the presence of 2×10^{-3}M ascorbic acid the apparent dissociation constant for noradrenaline was $2{\cdot}5 \times 10^{-3}$M.

Caffeine appeared to act as a competitive inhibitor of MAO activity with all the tested substrates and a 1/v *versus* caffeine plot with two tyramine concentrations ($S_1 = 1 \times 10^{-4}$M and $S_2 = 4 \times 10^{-4}$M) yielded an apparent K_i value of $1{\cdot}5 \times 10^{-3}$ according to the Dixon & Webb method (1960).

Uric acid behaved similarly to caffeine and an inhibition was verified with well-known MAO inhibitors such as ephedrine and iproniazid. Ascorbic acid reversed the inhibition by caffeine and also the effect of the other inhibitors tested. Fig. 1 shows a typical experiment.

The experiments were repeated with mitochondria isolated from the brain of the monkey (*Cercopitecus aetiops*) with the sucrose gradient technique of Marchbanks (1968) and the results paralleled those obtained with rat liver mitochondria. The possibility of a binding of labelled L-ascorbic[1-^{14}C]acid to the mitochondrial membrane in the presence of MAO substrates was studied parallel to the oxygraph experiments as a function of substrate concentration. The results of the L-ascorbic [1-^{14}C]acid binding and the stoichiometry between the amount of ascorbic acid bound and the extra amount of substrate oxidized in the presence of ascorbic acid are reported in Table 1. The inhibitory effect of caffeine on the binding is evident.

Chemical interaction between caffeine and ascorbic acid was tested in the partially hydrophobic solvent tris-dioxane (1:10) described previously by Galzigna (1970). A marked hypochromic effect induced by ascorbic acid on the 275 nm absorption band of caffeine was found which is consistent with a donor-acceptor type interaction

(Galzigna, 1969). An affinity constant of ascorbic acid for caffeine was determined spectrophotometrically (Galzigna & Rizzoli, 1970) and turned out to be 2 × 10^3M.

Burton (1951) recognized caffeine as an amino-acid oxidase inhibitor, and explained the inhibition in terms of an association of caffeine with the flavine coenzyme of the

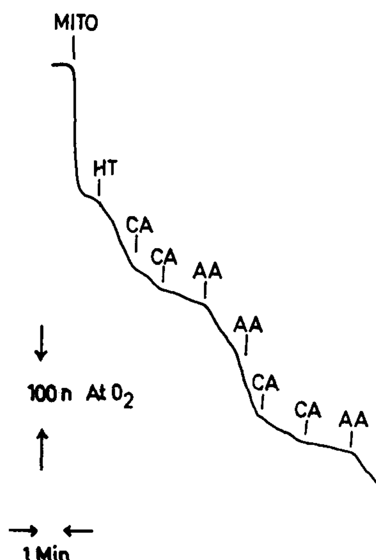


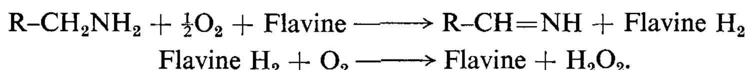
FIG. 1. Oxygraph tracing with MAO activity, caffeine inhibition and ascorbic acid reversal. 5-HT 1 × 10^{-3}M was used as a substrate and its addition is indicated with MT. Caffeine 5 × 10^{-}M indicated as CA and ascorbic acid indicated as AA were added where shown. Protein was 10·2 mg.

Table 1. *Binding of* L-*ascorbic* [1-^{14}C]*acid and stoichiometry between ascorbic acid bound and the extra amount of substrate oxidized in the presence of ascorbic acid.* Rat liver mitochondria were incubated at 22° in conditions similar to those used for the oxygraph experiments in the presence of L-ascorbic [1-^{14}C]acid. The purity of the sample was checked by t.l.c. in an amount corresponding to about 10 000 counts/min. After 10 min incubation the mitochondria were spun down at 6000 *g* for 15 min and 0·25 ml taken from the supernatant and transferred to 10 ml of the scintillation liquid (7 g of PPO, 0·6 g of DMPOPOP and 150 g of naphthalene in 1000 ml of dioxane). The residual radioactivity was then measured with a Packard liquid scintillation spectrometer (N.3320). Ascorbic acid average values from four experiments are reported.

Substrates	L-Ascorbic[1-^{14}C]acid bound (nmol min^{-1} mg^{-1})	Extra amount of substrate oxidized* (nmol min^{-1} mg^{-1})
Ascorbic acid	0	—
Ascorbic acid + caffeine	0	0
,, ,, + tyramine (1:1)	9·1	8·7
,, ,, + ,, (1:2)	12·7	11·5
,, ,, + ,, (1:5)	17·5	16·8
,, ,, + ,, (1:5) + caffeine ..	0	0
,, ,, + 5-HT (1:1)	12	12·2
,, ,, + ,, (1:2)..	16	15·3
,, ,, + ,, (1:5)	21	22·4
,, ,, + ,, (1:5) + caffeine	0	0

* Calculated from parallel MAO activity measurements.

oxidase. The same author gave the affinity constant of caffeine for flavine as $0{\cdot}1 \times 10^3$M. MAO has been recognized as a flavine bound enzyme (Erwin & Hellerman, 1967; Tipton, 1968) and therefore its inhibition by caffeine can be explained. In fact caffeine has been shown to be able to interact with aromatic electron donors by acting as an electron acceptor (Hanna & Sandoval, 1968) and a reduced flavine is known to be formed as a first step in the reaction sequence which constitutes the overall MAO activity:



$$R\text{–}CH_2NH_2 + \tfrac{1}{2}O_2 + \text{Flavine} \longrightarrow R\text{–}CH{=}NH + \text{Flavine } H_2$$
$$\text{Flavine } H_2 + O_2 \longrightarrow \text{Flavine} + H_2O_2.$$

Caffeine might compete with the oxygen for the reduced flavine, and ascorbic acid might reverse this effect by interacting with caffeine. In fact the affinity of ascorbic acid for caffeine is 20 times higher than the affinity of caffeine for flavine and 3 times higher than the affinity of caffeine for the mitochondrial MAO. The activating effect of ascorbic acid cannot be explained on the basis of a stimulation of the catalase system, since its action is not mimicked by reduced dichlorophenolindophenol or by uric acid which are also substrates for the catalase activity.

The binding of the labelled ascorbic acid in the presence of MAO substrates, its inhibition by caffeine and the stoichiometry between ascorbic acid bound and extra amount of substrate oxidized point to an effect of ascorbic acid as a true co-substrate of MAO or to its involvement in an allosteric regulation of the enzyme.

These results confirm that ascorbic acid must influence catecholamine metabolism at large and support its function in maintaining the normal metabolism of tyrosine (Knox, 1955). At molecular level ascorbic acid might influence the control mechanism involved in the binding and release of substrates from MAO (Hellerman & Erwin, 1968) and possibly protect MAO from the effects of a number of endogenous and exogenous toxic compounds which, by altering MAO activity, could upset (i.e. stimulate or depress) the normal functioning of the brain.

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