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# Short Communications

## The Effect of L-Ascorbate on Catecholamine Biosynthesis

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L-Ascorbate stimulates the enzymic hydroxylation of phenylalanine *in vitro* by recycling tetrahydrobiopterin, which reduces $O_2$ utilized in the reaction. It is suggested that ascorbate might have a similar function *in vivo*; this would explain the apparent regulation of tyrosine hydroxylase and tryptophan hydroxylase activities by this vitamin.

The enzymic hydroxylations of phenylalanine (Kaufman & Levenberg, 1959), tyrosine (Nagatsu *et al.*, 1964) and tryptophan (Renson *et al.*, 1961) involve the oxidation of 5,6,7,8-tetrahydrobiopterin to quinonoid dihydrobiopterin. In free solution both 5,6,7,8-tetrahydrobiopterin and quinonoid dihydrobiopterin form 7,8-dihydrobiopterin, and in a functioning enzyme system quinonoid dihydrobiopterin is recycled to tetrahydrobiopterin by dihydropteridine reductase and NADPH (Kaufman, 1964).

The above hydroxylations have been used to measure tetrahydrobiopterin, and in most cases the concentrations of tetrahydrobiopterin in the assay system has been maintained by enzymic reduction (Kaufman, 1970; Guroff *et al.*, 1967; Kaufman & Fisher, 1970). However, chemical reductants may also be used for this purpose, and these have the advantage that the possibility of contamination by dihydrofolate reductase, which reduces dihydrobiopterin to tetrahydrobiopterin, is lessened. The assay system thus becomes specific for tetrahydrobiopterin. Dithiothreitol is the reductant most commonly used, although it does cause some inhibition of the hydroxylase (Bublitz, 1969; Cotton, 1970). Monothiols, e.g. GSH and 2-mercaptoethanol, interfere with tyrosine determinations in many assay systems (Bublitz, 1969; Kaufman, 1959; Udenfriend & Cooper, 1952).

It has been suggested that high concentrations of L-ascorbate could regenerate tetrahydrobiopterin (Kaufman, 1959; Nielsen, 1969), although Bublitz (1969) found that L-ascorbate, at a concentration of 10mM, does not enhance the hydroxylation of phenylalanine. While developing a method for the determination of tetrahydrobiopterin in biological tissues we observed that the presence of ascorbate, added to prevent chemical oxidation of the extracted cofactor, markedly stimulated phenylalanine hydroxylase activity. The nature of this stimulation has now been studied, and a functional role for ascorbate *in vivo* is suggested.

Phenylalanine hydroxylase was prepared from the livers of 6-month-old female Sprague–Dawley rats as described by Kaufman & Fisher (1970). The purification was carried through the first two stages only, i.e. centrifugation and ethanol fractionation. The appropriate ethanol fraction was dissolved in buffer, to a concentration of 20.2mg of protein/ml, by using a solution of 0.2M-$Na_2HPO_4$ adjusted to pH6.4 with 0.1M-citric acid. Protein concentrations were determined by the method of Lowry *et al.* (1951), with dry bovine serum albumin [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] as standard.

Enzyme reactions were performed in the above buffer at 30°C with ascorbate (0.5$\mu$mol; adjusted to pH5.8 with 0.1M-NaOH), tetrahydrobiopterin (0.45nmol), catalase [Sigma (London) Chemical Ltd., Co. (4.0$\mu$g), L-[U-$^{14}$C]phenyl-alanine (The Radiochemical Centre, Amersham, Bucks., U.K.) (0.02$\mu$Ci; 477mCi/mmol), phenylalanine [Sigma (London) Chemical Co. Ltd.] (10.0nmol) and the enzyme (0.2mg of protein) in a total volume of 0.05ml. When the effect of adding supplementary amounts of components of the enzyme mixture was examined all incubation mixtures initially contained U-$^{14}$C-labelled phenylalanine (100nmol; 0.2$\mu$Ci), catalase (40$\mu$g), enzyme (2.0mg of protein) and buffer in a total volume of 0.4ml. Some incubation mixtures also contained tetrahydrobiopterin (27nmol), dihydrobiopterin (27nmol) and ascorbate (6$\mu$mol), as shown in Fig. 2. Supplements were added after 7min in a volume of 0.06ml as follows: for curve *A*, tetrahydrobiopterin (16nmol); for curve *B*, ascorbate (3.6$\mu$mol); for curve *C*, NADPH (3.4$\mu$mol); for curve *D*, enzyme (1.2mg of protein); for curve *E*, water. Samples of volume 0.04ml were removed throughout at 2min intervals for analysis. Reactions, started by the addition of enzyme and terminated with 0.01ml of 10% (w/v) trichloroacetic acid, were carried out in tubes of dimensions 1.2cm×7.5cm to allow an adequate supply

of $O_2$. A 0.03ml volume of solution containing phenylalanine (10mM) and tyrosine (10mM) was then added and the mixture centrifuged to remove precipitated protein. A 0.02ml portion of the supernatant was chromatographed overnight on Whatman 3MM paper with propan-2-ol – aq. $NH_3$ (sp.gr. 0.88) – water (8:1:1, by vol.) as solvent. Chromatograms were dried and amino acids were detected with ninhydrin. Areas on the chromatograms corresponding to the positions of phenylalanine and tyrosine were cut out, placed directly into scintillation vials and assayed for radioactivity by using a solution of 0.4% 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen (BBOT) [CIBA (A.R.L.) Ltd., Duxford, Cambridge, U.K.] in toluene as scintillation fluid. By this means over 97% of the radioactivity on the chromatograms was recovered. Results are calculated from the percentage of the total radioactivity recovered that appeared on the chromatogram in the position corresponding to that of tyrosine.

The enzyme preparation contained 20.2mg of protein/ml and had optimum pH5.95. No evidence was found for the presence of a protein factor that is reported to be present in crude hydroxylase preparations and that, by affecting a reversible association–dissociation of the enzyme, results in a pH optimum of 8.4 (Kaufman, 1970). $K_m$ values of $9.3\times10^{-4}$M for phenylalanine and $3.95\times10^{-6}$M for tetrahydrobiopterin were obtained. Concentrations of tetrahydrobiopterin above 1mM inhibited hydroxylation, but the reported inhibition by phenylalanine at concentrations above 2mM (Kaufman, 1970; Nielsen, 1969; Tourian *et al.*, 1969) was not observed.

Inhibition of the enzyme by ascorbate occurs at concentrations above 30mM (Fig. 1). However, the inhibition is much less than that reported to be caused by dithiothreitol (Bublitz, 1969), and a concentration of 270mM is required before activity is decreased by 50%.

The results shown in Fig. 2 show that hydroxylation in the absence of ascorbate stops before all the phenylalanine is utilized. Further addition of tetrahydrobiopterin restarts the reaction (curve *A*), whereas a further addition of enzyme does not (curve *D*). Hydroxylation is also restarted by the addition of ascorbate or NADPH (curves *B* and *C*). These results suggest that the reaction stops because of a lack of tetrahydrobiopterin and that ascorbate and NADPH will each generate more tetrahydrobiopterin from an intermediate. Recycling of tetrahydrobiopterin by ascorbate is further demonstrated by curve *F* for an experiment where the cofactor is used catalytically.

These results do not exclude the possibility that the hydroxylase enzyme used in this study contained a dihydropteridine reductase, which would utilize ascorbate in place of NADPH. Nevertheless care must clearly be taken when conclusions are drawn from experiments in which reduced nucleotides are shown to stimulate tyrosine formation by tissue extracts. Thus the argument by Jakubovic (1971) that such a stimulation 'suggests that dihydropteridine

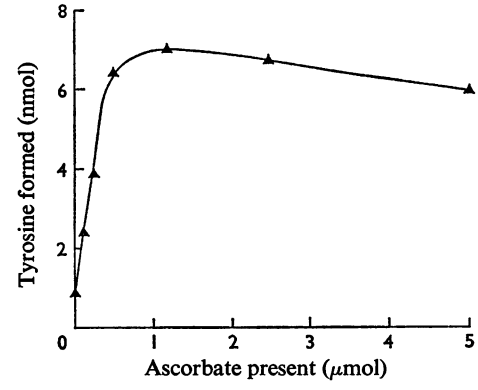


Fig. 1. *Effect of* L-*ascorbate on the activity of rat liver phenylalanine hydroxylase*

Reactions were carried out as described in the text.

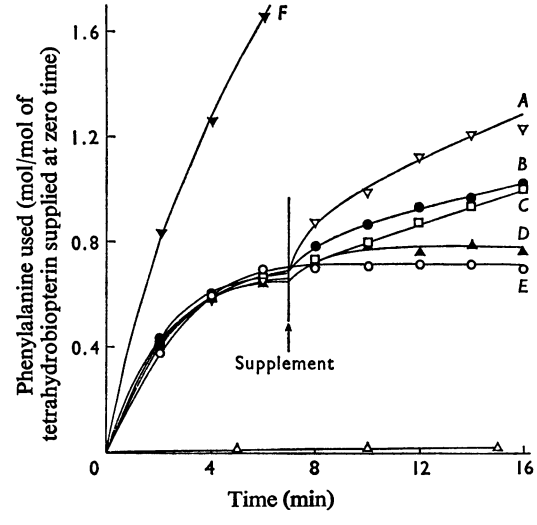


Fig. 2. *Effect of the delayed addition of ascorbate, NADPH and enzyme on the rate of tyrosine formation by rat liver phenylalanine hydroxylase*

The initial composition of reaction mixtures *A*, *B*, *C*, *D* and *E* was as described in the text. Reaction mixtures *F* and *G* also contained ascorbate, and reaction mixture *G* contained dihydrobiopterin in place of tetrahydrobiopterin. Supplements were added as follows: for curve *A*, tetrahydrobiopterin; for curve *B*, ascorbate; for curve *C*, NADPH; for curve *D*, enzyme; for curve *E*, water.

reductase is present by 11 weeks of an intra-uterine life' of a human foetus may be invalid.

Recycling of tetrahydrobiopterin by ascorbate allows the enzymic measurement of extremely small amounts of cofactor to be made, e.g. 30pmol of tetrahydrobiopterin/0.01 ml of solution. This is within the range of endogenous concentrations of cofactor reported in animal tissues (Guroff *et al.*, 1967), and amounts in less than 50mg of tissue can be measured (K. J. Stone & B. H. Townsley, unpublished work).

This effect of ascorbate *in vitro*, which is observed at concentrations comparable with those of some tissues, has particular importance when one considers the role of vitamin C in the body. The activities of tyrosine hydroxylase and tryptophan hydroxylase have been shown to be specifically influenced *in vivo* by the supply of ascorbate (Nakashima *et al.*, 1970), and the presence of *p*-hydroxyphenylpyruvate and homogenisate in the urine of scorbutic infants indicates incomplete oxidation of tyrosine. Similarly it has been shown that decreases in urinary adrenaline concentrations in premature infants with high serum tyrosine concentrations result from increased requirements for ascorbate and not directly from the raised serum tyrosine concentrations (Light *et al.*, 1971). As both tyrosine hydroxylase and tryptophan hydroxylase have a requirement for tetrahydrobiopterin, it is possible that some effects of ascorbate *in vivo* on these enzymes result from its capacity to maintain the pterin cofactor in a reduced form.

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