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## STABILITY OF ASCORBYL PALMITATE MOLECULE IN THE RAT BRAIN

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Recent investigations have shown the ability of ascorbyl palmitate (AP), a bioactive, lipid-soluble ester of ascorbic acid (AA), to penetrate neural tissues. This study seeks to determine the occurrence of hydrolysis of AP molecule in brain tissue, which could rather point to the action of AA alone carried over the biological barrier and then released from the AP compound. The integrity of AP molecule was examined qualitatively in the rat brain by thin-layer-chromatography. AP was injected into an internal carotid artery in a dose of 75 mg per rat after tying off the common and external carotid arteries at the same side. The rats were sacrificed 15 min later, the brain tissue was extracted with chloroform/methanol and chromatographed. The AP bands plated from the samples ipsilateral to the injection side strictly corresponded to the AP standard's location and were clearly separated from the AA standard with no overlap. The experiment showed that AP resists hydrolysis in the brain and thus the short-term biological effects of AP may be ascribed to the action of an intact ester molecule. The results may help elucidate the biological action of AP, a compound that increasingly attracts attention for biomedical use due to its antioxidant potential and ability to penetrate into the membrane signaling target sites.

*Key words: antioxidant activity, brain tissue, ascorbate, ascorbyl palmitate*

### INTRODUCTION

Ascorbyl-6-palmitate (AP) is a lipid-soluble ester of ascorbic acid (AA) or L-ascorbate and palmitic acid, in which the palmitate side chain is attached to the C6 position of the ascorbate ring. AP is considered safe and nontoxic and is often used as a food preservative (1). Although AP is a synthetic compound and does not exist endogenously, it appears to have biological activity. The compound

disperses in biomembranes, where it retains potent antioxidant properties of ascorbate (2). An *in vitro* electron spin resonance study with human blood loaded with equimolar concentrations of AP and AA showed that AP generates the ascorbyl radical signal the way AA does, in terms of the signal's shape, location, and increased amplitude with increasing concentrations (3). This attests to the scavenging power of AP being on par with that of AA. We previously found that AP crosses biological barriers, after oral ingestion in the cat, and accumulates in brain tissue (4). We also found in another study that AP interacts with the hypoxia-sensing process in that it enhances the hypoxic respiratory response in the cat (5).

Fairly extensive and varied biological activity of AP raises questions about the metabolism of AP once it enters the brain. The question we posed in the present study was of whether the AP molecule breaks down in neural tissue, thereby unleashing the action of ascorbate, or the molecule remains intact and then the ascorbate moiety would act being an integral part of the molecule. We addressed this issue by examining the integrity of AP molecule in brain tissue by means of thin-layer-chromatography after acute AP administration into the internal carotid artery.

## MATERIAL AND METHODS

### *Animal preparation and pharmacologic intervention*

The study and its protocol were approved by a local Ethics Committee. Two male Wistar rats weighing 255 g and 260 g were used for the study. The rats were housed in a 12 h/12 h light/night cycle and temperature-controlled (23°C) animal facility. The animals were anesthetized with  $\alpha$ -chloralose and urethane (35 and 800 mg/kg, ip, respectively), placed supine and were breathing spontaneously. The carotid artery bifurcation on the left side was surgically exposed. The common and external carotid arteries were ligated and a cannula was extended from an incision below the carotid bifurcation into the internal carotid artery. AP was dissolved in DMSO and was injected through the cannula, in a dose of 75 mg per rat, given in a volume of 0.3 ml. The injection was complete in 1-2 s and the syringe's cylinder was pressed down after the injection to prevent a rearward movement of the content. The rat was sacrificed 15 min after the injection of AP by intracardiac application of a saturated KCl solution to evoke cardioplegia. Then, the rat was decapitated, the cranium cut open, the entire brain removed, and the hemispheres separated sagittally.

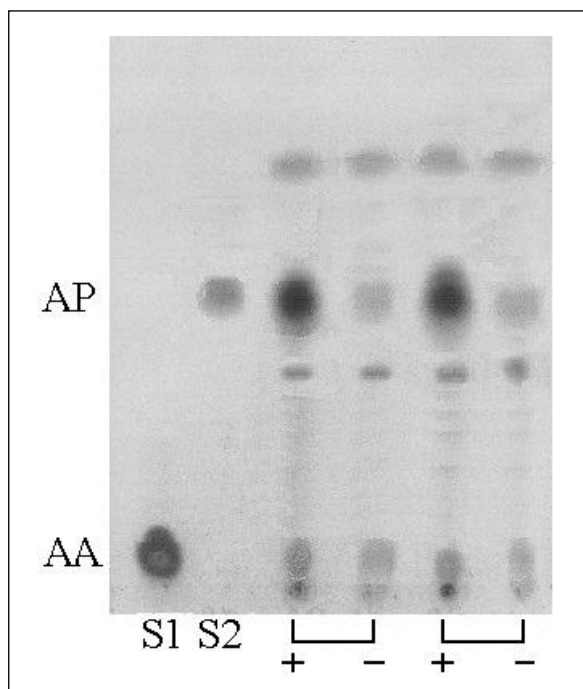
### *Brain tissue procedure*

The nature of the AP compound was studied with the use of thin-layer-chromatography (TLC). A brain hemisphere from the side contralateral to the injection side was used as a reference tissue. Tissue was extracted and homogenized in 5 ml of a chloroform/methanol mixture (2:1, v/v) in a glass homogenizer and then evaporated to dryness with anhydrous magnesium sulfate. The residue was dissolved in 2 ml of chloroform. During the procedure the tissue was protected from light. The same mixture was used for making stock solutions of standards, containing 1 mg·ml<sup>-1</sup> of AP and an equimolar amount of AA. Twenty microliter samples of both biological and standard materials were applied on precoated HPTLC silica gel 60 Å plates Merck KGaA (Darmstadt, Germany), using benzene:methanol:acetic acid (16:3:1, v/v/v) as solvents. The plates were developed with 3% FeCl<sub>3</sub> and 5% K<sub>3</sub>[Fe(CN)]<sub>6</sub> and the resolved spots were immediately photocopied.

## RESULTS

*Fig. 1* demonstrates the results of TLC screening and verification by standards of AP in the brain extracts from the two rats. The spots of the AA and AP standards (S1 and S2, respectively) were clearly separated from each other. In the hemispheres ipsilateral to the intracarotid AP injection side, the AP bands appeared at the level of the AP standard ('+' marks). Spots of AP were also traceable in the contralateral hemispheres ('-' marks). The  $R_f$  factor of the spots corresponding to the brain samples of AP-injected rats was nearly identical to that of the standard spot.  $R_f$  amounted to 0.465 for the AP standard. It was the same for the ipsilateral and contralateral samples in the first rat and was 0.470 and 0.460, correspondingly, in the second rat.

At the level of the AA standard, weak spots were traceable in the brain samples both ipsilateral and contralateral to the AP injection side, with a slightly higher intensity in favor of the former (*Fig. 1*).



*Fig. 1.* TLC of brain tissue extracts from two rats. S1 and S2 denote standards of ascorbic acid (AA) and ascorbyl palmitate (AP), respectively. The '+' and '-' marks correspond to the extracts from ipsilateral and contralateral hemispheres, respectively, relative to the intracarotid AP injection side in either rat. See Material and Methods for analytical details.

## DISCUSSION

Here we report that AP, injected into the internal carotid artery, penetrated the brain-blood barrier, as it was recovered from the brain, and was principally retained in brain tissue as an intact molecule. The TLC analysis we used has

limitations in that it is a qualitative method and no conclusion can be drawn as to the amount of AP that permeated through the barrier or a fraction of it that underwent hydrolysis in the brain. The probability of the compound's hydrolysis may be inferred from the more intense spots at the level of the AA standard on the side ipsilateral to AP injection (*Fig. 1*), which may correspond to the ascorbate moiety being plated in that region after being dissociated from the AP molecule. However, AP hydrolysis in the brain seems rather minor in the time span of 15 min from AP injection to sacrificing the animal, used in the experimental protocol of the present study.

There are endogenous lipase-esterase catalytic systems present in neuronal membranes (6). Therefore, release of ascorbate from the AP molecule is not an unlikely assumption, although it has been unclear whether these catalytic systems would be effective against a synthetic ester that enters neural tissue. The present experiment shows that AP is rather resistant to hydrolysis *in vivo*, at least in the short-term. This result is in accord with the observation that AP survives, as an intact molecule, osmotic lysis and centrifugation washes in erythrocyte membranes (2).

The results of the present experiment, when extrapolated to our previous work that showed enhancement of the hypoxic respiratory response by AP (5), indicate that the short-term biological effects of AP may be ascribed to the action of a whole molecule. The question, however, still remains unresolved of whether the AP molecule, as such, or its ascorbate moiety is integral to these effects. The resolution of this issue requires alternative study designs. With regard to the antioxidant action of AP, Ross et al (2) reported that the AP molecule spreads through the plasma membrane of erythrocytes in such a manner that the fatty acid portion is intercalated into its outer and the ascorbate portion into inner bilayers. In this dimensional scheme of spreading, the AP molecule remains intact and its ascorbate portions sustains the antioxidant activity.

Biological activity and physicochemical properties of AP make it a compound of apparent research interest. AP is lipophilic and thus apt to permeate through biological barriers. It readily penetrates the blood-brain barrier and is the preferred form of AA transport into neural tissues (4). AA may then act in membrane lipid bilayers, otherwise inaccessible to this water-soluble compound, which incidentally are the target sites of many a signaling molecule. Ascorbate's antioxidant action is expanded to these vulnerable to free radical attacks cell membrane aspects, which may result in the modulation of signaling processes. This may be exemplified by the enhancing effect of AP on the carotid body-mediated hypoxic ventilatory response (5).

In conclusion, AP shows its biological activity as an intact ester molecule. Our results represent a further step in the elucidation of biological properties of AP. We believe that investigation of AP action has a bearing on its increasing use as a food preservative or additive to multivitamin preparations. That also includes a

careful contemplation of the potential risks posed by a compound that is capable of penetrating the brain.

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