

Vitamin E Phosphate: An Endogenous Form of Vitamin E

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Summary

α -Tocopherol is widely recognized as an anti-oxidant of considerable biological importance¹. To date only free and protein-bound or associated α -tocopherol has been detected in biological tissues². Here we show that the monophosphate ester of α -tocopherol, α -tocopheryl phosphate (TP), is a naturally occurring, water-soluble form of vitamin E. We also show that TP has not been detected previously because standard methods of purifying α -tocopherol do not also purify TP. Furthermore, supplementation of TP in rats (30 mg/kg) daily for 32 days, resulted in a significant increase in TP levels (62%) in liver, compared to control (no TP supplementation; $p < 0.05$). Importantly, α -tocopherol levels in the liver tissues were significantly increased (40%; $p < 0.05$), indicating the possible conversion of the monophosphate ester form, TP, to α -tocopherol.

Introduction

α -Tocopherol is recognized as an active form of vitamin E¹. It is a potent free radical scavenger with a vital role in the maintenance of cellular integrity through its capacity to protect the polyunsaturated fatty acyl moieties of phospholipids in biological membranes³ and plasma lipoproteins^{4,5,6,7}. Paradoxically, vitamin E is also a pro-oxidant and a potential source of free radicals⁸. However, it is not evident how the storage and transport of α -tocopherol in biological systems can be achieved without the generation of free radicals. It is our hypothesis that α -tocopherol exists in an unrecognised form to meet these conflicting requirements.

Tocopheryl phosphate has not been reported previously as a natural form of vitamin E. Here we describe a method developed to allow its detection and assay. We also propose that since this additional form has now emerged, previous amounts of vitamin E in plants and animals have been underestimated.

Materials and Methods

Synthesis of compounds. All reagents except d- α -tocopherol were of analytical grade. α -Tocopheryl phosphate was synthesized by first reacting natural α -tocopherol with phosphoric anhydride at 70-85°C with high shear. The tocopheryl pyrophosphates resulting in the mixture were hydrolysed at 100°C with 2 moles of water per tocopherol to give a mixture of tocopheryl phosphate (TP), di-tocopheryl phosphate (T₂P) and phosphoric acid.

The sodium salt form of TP (Na₂TP) was prepared as follows: sodium hydroxide (50%), equimolar to the phosphoric acid, was then added and the mixture dispersed in ethanol. The insoluble sodium phosphate was removed by centrifugation. Then a further 50% sodium hydroxide, equivalent to 2 moles per mole of tocopherol, was added to precipitate the disodium TP that was recovered by centrifugation and evaporated to dryness. The mixture was analysed by P³¹NMR.

Animals. Male Sprague-Dawley rats (250-390 grams) were fed standard laboratory rat pellets (Glenforest Stockbreeders, Perth, Western Australia) and either water, or water containing TP-sodium form (30 mg/kg) *ad libitum* for 32 days.

Extraction of TP and analysis from biological tissues and foods. TP-enriched fractions were obtained by a dichloromethane extraction method developed in our laboratory. Briefly, a 1 gram samples was homogenised in 10 mL dichloromethane (DCM), centrifuged, the upper layer of denatured (proteinaceous) material discarded, the lower liquid-phase collected, and then dried down under nitrogen gas. Quantification of TP in the sample was done through the use of an appropriate internal standard (e.g. dicetyl phosphate; DCP) into the homogenised mixture of tissue plus DCM, re-homogenising, centrifuging and drying the DCM-phase, as above. Nine mL 2M KOH was added and incubated at 80°C for 40 min, with stirring. After this saponification step 10 mL hexane was added, samples shaken vigorously, centrifuged and the upper phase discarded. Ten mL 2M HCl was then added to the remaining aqueous phase to bring the pH to less than 1 (checked with pH test strips; Merck, cat. no. 1.09540). Ten mL hexane was then added, the samples shaken vigorously, centrifuged, and the upper phase placed into glass vials and dried down under nitrogen gas. This formed the TP-rich fraction. Samples were stored at -80°C until they were analysed by electrospray mass spectrometry (ESMS). Samples were dissolved in 1 mL THF containing 1% NH₃, 20 mL injected into the ESMS (Micromass Platform), with the

Table 1. α -Tocopherol and α -tocopheryl phosphate levels detected in biological tissues.

| Tissue | | α -Tocopherol phosphate ($\mu\text{g/g}$) | α -Tocopherol ($\mu\text{g/g}$) |
|----------------|--|--|--|
| Liver | Rat (adult male; SD) n=15 | 11 – 13 | 6 – 8 |
| | Guinea pig (adult male; tricolour) n=4 | 6 – 10 | 12 |
| | Pig (young male; commercial) n=4 | 15 – 21 | 3 – 9 |
| | Chicken (2 month old male) n=14 | 31 – 41 | 5 – 24 |
| Adipose tissue | Guinea pig (adult male; tricolour) n=4 | 38 - 42 | 30 |
| | Rat (adult male; SD) n=9 | 26 – 30 | 115 |
| | Human (adult female) n=3 | 22 - 28 | 25 |

ESMS was convenient for the analysis of TP. α -Tocopheryl phosphate is readily ionisable and is soluble in water and inorganic solvents at low pH; these are properties that are ideally suited to ESMS. With the addition of a suitable internal standard, levels of TP in tissues can be routinely determined by ESMS.

Our findings that TP is resistant to hydrolysis and oxidation, coupled with the requirement to liberate free α -tocopherol by alkaline hydrolysis during the extraction process, explains why the α -tocopherol present as TP had not been previously detected using the standard assays for vitamin E. Reported amounts of ‘total α -tocopherol’ present in samples will consequently have always been underestimated.

Using our extraction method and ESMS analysis, the TP content in a range of biological tissues and foods were determined and are shown in tables 1 and 2.

Table 2. α -Tocopherol and α -tocopheryl phosphate levels detected in foods.

| Sample | α -Tocopheryl phosphate ($\mu\text{g/g}$) | α -Tocopherol ($\mu\text{g/g}$) |
|-----------------|--|--|
| Wheat germ | 22 | 1190 |
| Sunflower seeds | 9 | 500 |
| Almonds | 5 | 270 |
| Wheat germ | 10 | 110 |
| Olive oil | 5 | 62 |
| Chocolate | 237 | 27 |
| Spinach | 3 | 18 |
| Cheddar | 337 | 17 |
| Brie | 437 | 14 |
| Oat bran | 4 | 13 |
| Peanuts | 21 | 7 |
| Peas | 3 | 6 |
| Broccoli | 5 | 5 |
| Apple | 2 | 3 |

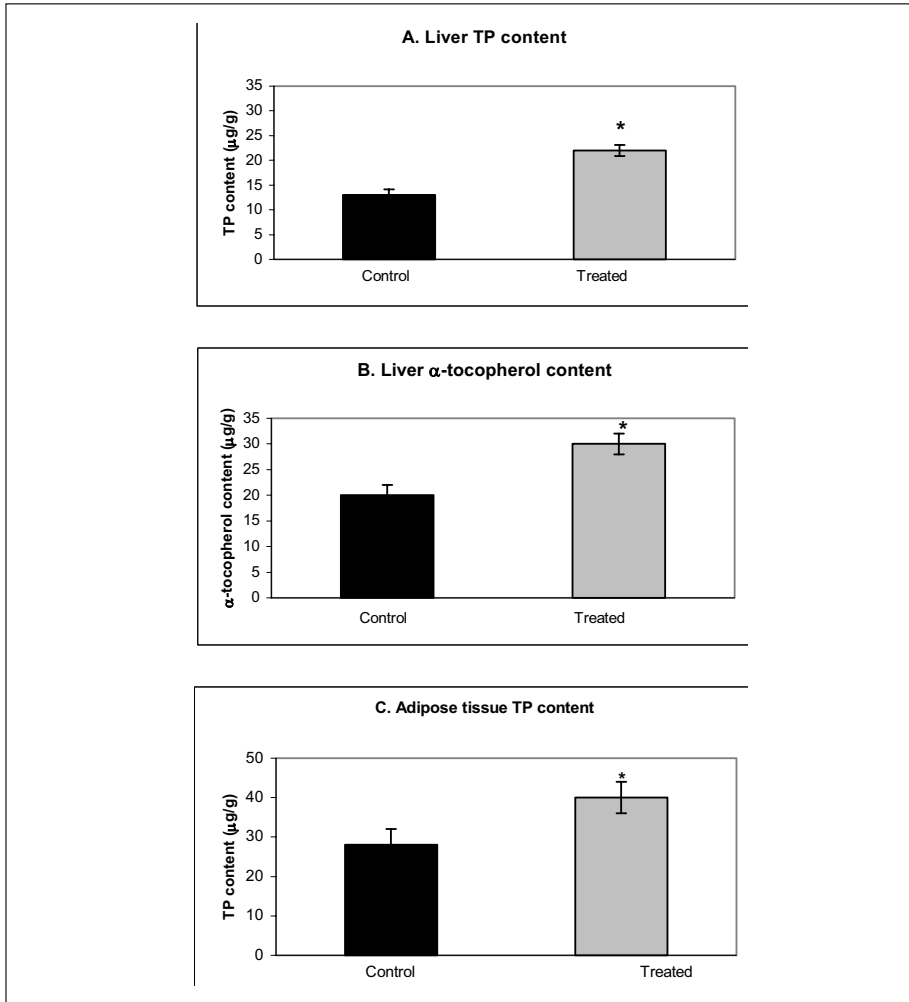


Figure 2. α -Tocopherol and TP content in liver and adipose tissue following 32 day TP supplementation (30 mg/kg).

Furthermore, figure 2 shows that supplementation of TP in rats (30 mg/kg body weight) daily for 32-days, resulted in a significant increase in TP levels in liver (62%) and adipose tissue (50%), compared to control (no TP supplementation; $p < 0.05$). Interestingly, α -tocopherol levels in the liver tissues were significantly increased (40%; $p < 0.05$) in TP supplemented animals, indicating the possible conversion of the monophosphate ester form, TP to α -tocopherol. Studies are underway to understand the biological significance of TP, and its relation to the well-characterized α -tocopherol.

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For more information please visit www.vitalhs.com

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