

Short communication

### THE ANTIOXIDANT PROPERTIES OF CARNITINE *in vitro*

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**Abstract:** Many of the effects of carnitine are ascribed to its antioxidant properties. The aim of this study was to evaluate the antioxidant properties of carnitine *in vitro*. Carnitine was found to decolorize ABTS<sup>•+</sup>, and to protect fluorescein against bleaching induced by AAPH-derived peroxy radicals and peroxy nitrite, thiol groups against oxidation induced by hydrogen peroxide, peroxy radicals, hypochlorite and peroxy nitrite, and erythrocytes against hemolysis induced by peroxy radicals and hypochlorite. These results show that carnitine has a direct antioxidant action against physiologically relevant oxidants.

**Key words:** Antioxidant, Carnitine, Hydrogen peroxide, Hypochlorite, Peroxy radical, Peroxy nitrite

### INTRODUCTION

L-Carnitine ( $\beta$ -hydroxy- $\gamma$ -4-*n*-trimethylaminobutyric acid) is an important metabolite that performs many functions in the body. Its main metabolic role is the transport of long-chain fatty acids into the mitochondrial matrix across the inner mitochondrial membrane. It is also a cofactor in the peroxisomal oxidation of very long-chain fatty acids, and it plays a role in the central nervous system,

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Abbreviations used: AAPH – 2,2'-azobis(2-amidinopropane) dihydrochloride; ABTS – 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate); DTNB – 5,5'-dithio-bis(2-nitrobenzoic acid); PBS – phosphate-buffered saline

mediating the transfer of acetyl groups for acetylcholine synthesis and affecting signal transduction pathways and gene expression. It intensifies respiration and peroxisome function and is often used as a food additive supposed to increase physical performance [1, 2], promote weight loss [3, 4] and increase reproductive performance [5, 6]. It has been also found to improve immunological functions [7, 8] and is suggested to be a therapeutic agent for several neurodegenerative disorders [9, 10].

The beneficial effects of carnitine appear to be mediated, at least in part, by its antioxidant properties, which include the upregulation of the level of glutathione [8, 9] and the elevation of the activities of antioxidant enzymes [8]. The *in vivo* effects may be mediated by the direct antioxidant action of carnitine or by its effects on the biosynthesis of antioxidant enzymes and other proteins. In this context, it is of interest to characterize the antioxidant properties of carnitine in well-defined systems *in vitro*, as this may contribute to the elucidation of the mechanisms of the antioxidant action of this compound. Previous studies have demonstrated that carnitine reacts with superoxide and hydrogen peroxide, has reducing properties, chelates iron, and prevents lipid peroxidation and DNA cleavage [11, 12]. The aim of this study was to further characterize the antioxidant properties of carnitine *in vitro*, particularly with regard to pathophysiologically relevant oxidants such as peroxynitrite and hypochlorite. These oxidants are not neutralized enzymatically *in vivo*, so low-molecular weight antioxidants that would protect against their action would be even more important than ones protecting against superoxide or hydrogen peroxide, which are removed enzymatically.

## MATERIALS AND METHODS

### Reagents

The reagents were obtained from Sigma/Aldrich (Poznań, Poland) unless stated otherwise. Peroxynitrite was synthesized from sodium azide and ozone according to the method of Pryor *et al.* [13] with minor modifications [14]. This method yields low ionic strength peroxynitrite that does not contain hydrogen peroxide as an impurity. Briefly, an ozone stream from an ozonator (75 µg/ml in oxygen, 100 ml/min) was bubbled for about 60 min through a glass-frit into 100 ml of 0.2 M sodium azide in water that had had its pH adjusted to 12 with 1 N NaOH and had been chilled to 0°C in an ice-water mixture. The final concentration of peroxynitrite was about 70-90 mM. Stock solutions were stored at -85°C and used within 4-6 weeks after synthesis. Before each experiment, the concentration of peroxynitrite was estimated spectrophotometrically at 302 nm in 0.1 M NaOH ( $\epsilon_M = 1670 \text{ M}^{-1}\text{cm}^{-1}$ ).

### Reactivity with ABTS<sup>•+</sup>

We used a slight modification [15] of the methods of decolorization of the ABTS<sup>•+</sup> radical [16]. ABTS<sup>•+</sup> was prepared via a reaction of 19.5 mg of

ammonium 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate) with 3.3 mg of potassium persulfate in 7 ml of 100 mM sodium phosphate buffer, pH 7.4, for 12 to 16 h in the dark. The thus-obtained dark blue-green solution of ABTS<sup>•+</sup> was aliquoted and kept frozen at -20°C until use. The aliquots were thawed and diluted with the same buffer to give an absorbance of 1.0 at 414 nm immediately before use. Aliquots of solutions of the tested compounds (10 µl) in the phosphate buffer were mixed rapidly with 990 µl of the diluted ABTS<sup>•+</sup> solution in a spectrophotometric cuvette, and the absorbance was measured exactly 3 min after mixing the antioxidant(s) with the ABTS<sup>•+</sup> solution.

#### **The protection of fluorescein against oxidant-induced bleaching**

Fluorescein (2 µM) in 100 mM phosphate buffer, pH 7.4, was mixed in the presence or absence of the tested antioxidants, with oxidants, namely 20 µM HOCl, ONOO<sup>-</sup> or 10 mM AAPH (final concentrations), in a final volume of 1 ml. The fluorescence of the samples was measured at the excitation/emission wavelengths of 480 nm/514 nm in a Hitachi F-2500 spectrofluorimeter after a 15-min incubation with HOCl or ONOO<sup>-</sup> at room temperature, or after a 60-min incubation with AAPH at 37°C. The results were analyzed in a way analogous to that presented by Kohri *et al.* [17] by plotting  $\Delta F/\Delta F_0 - 1$  vs the concentration of the antioxidant, where  $\Delta F$  is the decrease in fluorescence induced by an oxidant in the presence of a given concentration of that antioxidant, and  $\Delta F_0$  is the decrease in fluorescence induced by the same amount of the oxidant in the absence of an antioxidant. The initial part of this curve is linear (Robaszkiwicz *et al.*, submitted).

#### **The protection of thiol groups against oxidation**

Glutathione solution (250 µM, final) in phosphate-buffered saline (145 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4; PBS) was mixed in the presence or absence of the tested antioxidants, with oxidants, namely 0.5 mM H<sub>2</sub>O<sub>2</sub>, 10 mM AAPH, 375 µM HOCl or 500 µM ONOO<sup>-</sup> (final concentrations) to make up a volume of 500 µl. After a 15-min incubation at room temperature (hypochlorite and peroxynitrite) or a 1-h incubation at 37°C (H<sub>2</sub>O<sub>2</sub>, AAPH), the samples were diluted with 1 ml of 0.1 M phosphate buffer, pH 8.0, and added with 10 µl of 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). The absorbance of the thionitrobenzoic acid was measured at 412 nm.

#### **The protection of erythrocytes against oxidant-induced hemolysis**

Aliquots of erythrocyte suspensions in phosphate-buffered saline containing no oxidant or supplemented with antioxidants were added with an oxidant (30 mM AAPH or 375 µM hypochlorite) to a final volume of 200 µl. The changes in their turbidity were measured at 600 nm in an Anthos 2010 absorbance reader after a 15-min incubation at room temperature for hypochlorite, or after various times of incubation at 37°C for AAPH. The initial concentration of erythrocytes was chosen to provide an initial turbidity for the suspension of 1.0. All the presented results are mean values from at least 3 independent experiments.

## RESULTS AND DISCUSSION

Carnitine reduced  $\text{ABTS}^{\bullet+}$  like other antioxidants and was 3.6-fold less efficient than Trolox in this reaction (Fig. 1). Carnitine did not exhibit any reactivity in the FRAP assay (giving values lower than the reagent blank), in agreement with its reported ability to chelate iron ions [11].

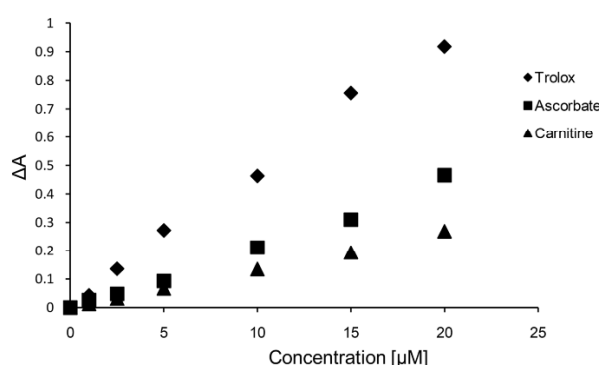


Fig. 1. The reactivity of carnitine in the  $\text{ABTS}^{\bullet+}$  decolorization assay. The decrease in the absorbance of preformed  $\text{ABTS}^{\bullet+}$  due to antioxidants was measured at 414 nm after 3 min of reaction.

$\text{ABTS}^{\bullet+}$ , although widely used in studies of antioxidant activity, is a promiscuous and not a naturally occurring radical. The reactivity of carnitine against more relevant oxidants is therefore even more interesting. We compared the reactivities of carnitine and standard antioxidants with respect to other oxidants. Carnitine effectively protected fluorescein against bleaching induced by hypochlorite, peroxynitrite and peroxy radicals derived from the commonly used azo initiator, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH),

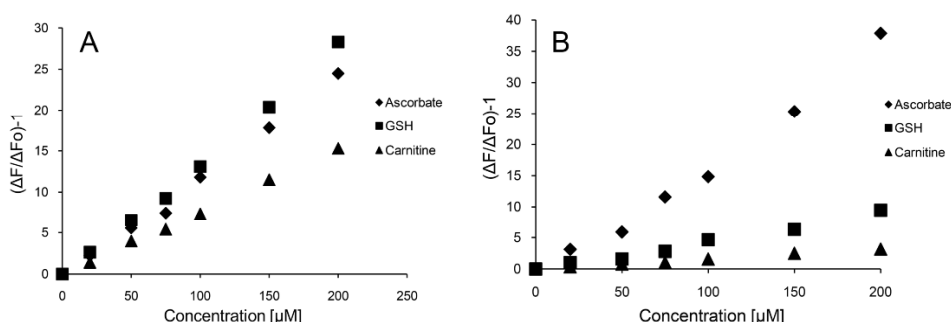


Fig. 2. The protection of fluorescein against AAPH-induced (A) and peroxynitrite-induced bleaching (B) by antioxidants. Fluorescein (2 μM) reacted with AAPH (10 mM) at 37°C for 1 h or with 20 μM peroxynitrite at room temperature for 15 minutes in 0.1 M sodium phosphate, pH 7.4, in the absence or in the presence of antioxidants at various concentrations. The decreases in fluorescence ( $\Delta F_0$  and  $\Delta F$ , respectively) were measured at 480 nm/514 nm.

although its protective activity against peroxynitrite was distinctly lower with respect to ascorbate and glutathione (Fig. 2). Surprisingly, in contrast to glutathione and ascorbate, carnitine was unable to protect against hypochlorite-induced fluorescein bleaching, even slightly enhancing the loss of fluorescence evoked by hypochlorite (not shown).

Another test reaction for antioxidant activity was assessing the protection of thiol groups against oxidation induced by oxidants. In this system, carnitine protected thiol groups against oxidation induced by hydrogen peroxide, AAPH, hypochlorite and peroxynitrite. It was more effective than Trolox at protecting against AAPH-derived radicals and hypochlorite (Fig. 3).

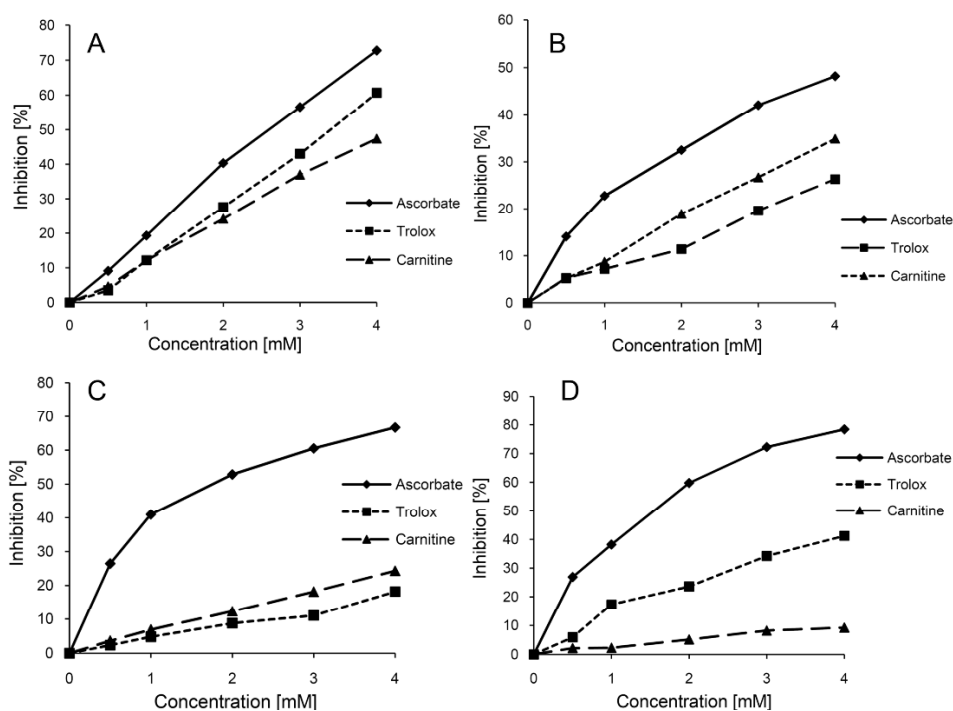


Fig. 3. The inhibition of the  $H_2O_2$ - (A), AAPH- (B), hypochlorite- (C) and peroxynitrite-induced (D) thiol group oxidation of glutathione by antioxidants. Glutathione (250  $\mu$ M) in PBS, pH 7.4, was incubated with 0.5 mM hydrogen peroxide, 10 mM AAPH, 375  $\mu$ M HOCl or 500  $\mu$ M ONOO<sup>-</sup> (final) in the absence and presence of antioxidants at 37°C for 1 h (A, B) or 15 min at room temperature (C, D). The concentration of -SH groups was then determined immediately with Ellman reagent. In the absence of antioxidants, 90.1% (A), 63% (B), 72.6% (C) and 48.6% (D) of the thiol groups were oxidized.

Treating erythrocytes with oxidants induces hemolysis, which can be easily monitored turbidimetrically in a plate reader, and which is prevented by antioxidants. In this system, carnitine protected erythrocytes against lysis induced by AAPH and hypochlorite (Fig. 4).

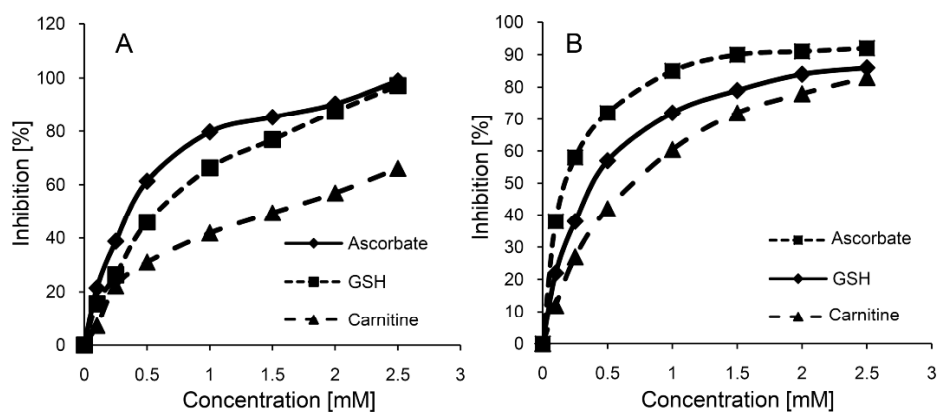


Fig. 4. The protection of erythrocytes against AAPH- (A) and hypochlorite-induced (B) hemolysis by antioxidants. Erythrocytes were incubated in PBS with 30 mM AAPH at 37°C for 4 h (A) or with 375  $\mu$ M hypochlorite for 15 min (B), and hemolysis was evaluated by assessing the decrease in the turbidity of the suspension at 600 nm. The turbidance of the control samples was 1, that of the AAPH-treated samples without antioxidants was 0.45, and that of the hypochlorite-treated samples without antioxidants was 0.22.

It has been demonstrated by other authors that L-carnitine reacts with superoxide and hydrogen peroxide. It has reducing activity comparable to that of the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) stable free radical and ferricyanide ion. This compound protects linoleic acid against autoxidation and peroxidation induced by UV-photolysis of  $H_2O_2$ , and pBR322 plasmid DNA against cleavage induced by UV-photolysis. It is an efficient scavenger of ferrous ions, more potent than ferrozine [11, 12].

The results of this study fully confirm previous findings on the antioxidant properties of carnitine *in vitro*. In specific cases (*viz.* fluorescein oxidation by hypochlorite), the possibility of aggravating damage cannot be excluded (as with any antioxidant). A similar pro-oxidant effect of carnitine has been reported under specific conditions of microsomal lipid peroxidation [18].

The relative reactivity of carnitine with respect to the tested reference antioxidants was different for the various systems, which is understandable taking into account the complexity of antioxidant action. I. a., antioxidants may react directly with oxidants, break oxidant-induced reactions, or repair oxidatively damaged molecules [19]. The contributions of these effects may be different in different systems. This study expands on previous findings by demonstrating the ability of carnitine to counteract the oxidative effects of the physiologically relevant oxidants: hydrogen peroxide, peroxy radicals, hypochlorite and peroxynitrite.

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