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Albumin, the responsible protein of the Cu^{2+}-dependent hydrolysis of O-hexyl O-2,5-dichlorophenyl phosphoramidate (HDCP) by chicken serum "antagonistic stereoselectivity"

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ABSTRACT

O-hexyl O-2,5-dichlorophenyl phosphoramidate (HDCP) is a chiral analogous compound of the methamidophos insecticide that induces delayed neuropathy, and the R-(+)-HDCP enantiomer is an inhibitor of neuropathy target esterase (NTE). This enantiomer is not hydrolized by Ca^{2+}-dependent phosphotriesterases in mammal tissues. Our group had reported R-(+)-HDCP hydrolysis in chicken serum enhanced by 30–250 μM copper in *ex vivo* assays, which we call "antagonistic stereoselectivity". We checked the hypothesis of the role of cupper binding proteins. Two hundred micrograms of human serum ceruloplasmine or horse kidney methallotionein in 1 mL containing 400 μM HDCP for 60 min showed no significant Cu^{2+}-dependent hydrolysis. However under the same conditions, 10 μL of chicken serum or 10 μL of buffer containing 216 μg of chicken serum albumin (CSA) (amount of albumin content in this serum volume) with 100 μM Cu^{2+} showed the same stereoselectivity and similar levels to the Cu^{2+}-dependent R-(+)-HDCP hydrolysis. About 75% of R-(+)-HDCP were hydrolyzed after 120 min in the presence of 100 μM Cu^{2+} (inhibited by 5 mM EDTA). No effects was observed by divalent cations Cu^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+} and Mg^{2+}. These results confirm that albumin is the protein responsible for "antagonistic stereoselectivity" observed in chicken serum.

1. Introduction

Organophosphrus hydrolyzing enzymes, such as phosphotriesterases, including paraoxonase, are considered to play a critical role in the *in vivo* detoxication of organophosphorus compounds (OPs), and for their potential application in treatments of OP intoxications (Sogorb et al., 2004a). Albumin's esterase activity hydrolyzing OPs have been reported (Ortigoza-Ferado et al., 1984; Furlong et al., 1988; Lockridge et al., 2008; Li et al., 2008; Jiang et al., 2013). Its detoxication capacity of OPs has been demonstrated (Sogorb et al., 1993, 1998, 2002, 2004b, 2007) as well as the formation of adducts with OPs which can be used as biomarker of exposure (Marsillach et al., 2013; Li et al., 2013).

O-hexyl O-2,5-dichlorophenyl phosphoramidate (HDCP) is an analogous chiral compound to the methamidophos insecticide. This compound belongs to the o-diclorophenyl phosphoramadates series, which was designed and synthesized to elucidate the molecular mechanisms of inhibition and aging of acetylcholinesterase (AChE) and neuropathy target esterase (NTE). Both type B-esterases of the nervous system have been associated with acute neurotoxic syndromes known as cholinergic and organophosphate-induced delayed polyneuropathy (OPIDP), which induces the methamidophos insecticide (Vilanova et al., 1987). The first *ex vivo* studies conducted by Vilanova et al. (1987) and Johnson et al., 1989), which used chicken brain homogenate (the usual OPIDP model) and racemic HDCP, have demonstrated that this phosphoramidate is a more powerful inhibitor of NTE than of AChE, but does not induce the aging reaction.

In vivo studies using adult chickens have demonstrated that administering the same racemic HDCP mixture induces OPIDP syndrome through the inhibition and aging of brain NTE. This *ex vivo versus in vivo* experimental controversy has considered the hypothesis of a possible *in vivo* stereoselective metabolism by type A-esterases, also known as

Abbreviations: OPs, organophosphorus compounds; NTE, neuropathy target esterase; HDCP, O-hexyl O-2,5-dichlorophenyl phosphoramidate; HDCPase, O-hexyl O-2,5-dichlorophenyl phosphoramidate hydrolyzing activity; AChE, acetylcholinesterase; OPIDP, organophosphate-induced delayed polyneuropathy

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phosphotriesterases, as so "the HDCP enantiomer, the most powerful inhibitor of NTE, which does not induce the aging reaction of brain NTE in *ex vivo*, must be metabolized by systemic A-esterases *in vivo*". Later in an *in vitro* study conducted on NTE using chicken brain homogenate and purified (99%) HDCP enantiomers by chiral chromatography (HPLC), identified in their chemical structure (polarized light rotation and IPAD rules), Sogorb et al. (1997) demonstrated that the R-(+)-HDCP enantiomer induced the inhibition and aging of brain NTE at levels above 80%, while the S-(–)-HDCP enantiomer inhibited NTE, but did not age it because NTE levels of 90–100% were recovered. Moreover, studies into the *ex vivo* metabolism of HDCP in several bird and mammal tissues have demonstrated the Ca^{2+}-dependent (paraoxonase-1) stereoselective hydrolysis of isomer S-(-)-HDCP by the main tissues responsible for systemic metabolism, such as the liver, serum and kidney (Díaz-Alejo et al., 1998: Monroy-Noyola et al., 1999a: 1999b: 2007:, 2015). These studies, done using type A and B esterases, reinforce the neurotoxic stereoselective HDCP hypothesis; RSo(+)-HDCP is the isomer that is not degraded by the organs and tissues responsible for metabolism by accessing the brain to induce the delayed neurotoxic effects of HDCP.

One surprising observation has been made about the stereoselective hydrolysis of R-(+)-HDCP, which is about 20-fold greater and contrary to that observed in the presence of Ca^{2+} in several bird tissues, which we called "antagonistic stereoselectivity" as it mainly hydrolyzes the other enantiomer that differs from that preferentially done by Ca^{2+}-dependent activity in liver extracts (Monroy Noyola et al., 2017). The present study presents evidence that the protein responsible for such Cu^{2+}-dependent activity in chicken serum is albumin, and not other copper-binding proteins like metallothionein or ceruroplasmine.

2. Materials and methods

2.1. Chemicals

HDCP (O-hexyl O-2,5-dichlorophenyl phosphoramidate, purity ca. 95%) was supplied by Dr. Naumman (Bayer Chemical Company, Germany). Chicken serum, horse serum ceruloplasmine, human kidney metallothionein, chicken serum albumin, Coomassie Brillant Blue G-250, TRIS, calcium chloride, EDTA, copper sulfate, zinc chloride, ferric sulfate, manganese chloride, magnesium chloride, and other chemicals of reactive grade used in the proteins quantification were purchased from Sigma Chemicals Company. Hexane, 1,2-dichloroethane and ethanol HPLC grades came from Scharlau Chemie S.A.

2.2. Albumin estimation in chicken serum

The amount of protein in chicken serum was determined by the Bradford method (1976), adapted to a microassay using an automated station (Beckman Coulter Biomek 1000). On a 96-well microplate, 10 µL chicken serum samples were mixed with 60 µL of 10 mM Tris buffer, pH 7.0, and 180 µL of Bradford reagent solution. A calibration curve was designed with bovine serum albumin. Absorbance at 660 nm was recorded with the photometric reading unit and the results of the determinations made with this equipment were processed using the Biodata software. Having quantified the total proteins in chicken serum (100%), they were estimated to be 60%, which corresponded to the albumin concentration.

2.3. HDCP hydrolysis chicken serum and cuproproteins

HDCP stereoselective hydrolysis was measured by the chiral HPLC method [Díaz-Alejo and Vilanova 1993]. It designated the (+)-HDCP and (−)-HDCP stereoisomers, which were eluted in first and second places from the chromatographic system, based on a polarized light study with HDCP pure enantiomers (99.5%) isolated by the same chromatographic procedure. According to the Cahn-Ingold-Prelog nomenclature rules, the specific R/S configuration was established by comparing it with the properties of NTE phosphorylated with R-(+)-O-n-hexyl-S-methylphosphorothioamidate (Sogorb et al., 1997). The quantification of the remaining concentration of both the HDCP enantiomers from this chromatographic method was previously used by our research group in enzymatic studies into hydrolysis (Díaz-Alejo et al., 1998; Monroy-Noyola et al., 2015, 2017). Ten microliters of chicken serum, or 10 µL of buffer containing 200 µg of ceruloplasmine or metallotionein or 216 µg of chicken serum albumin, were incubated with 1 mL of substrate solution containing 400 µM of the HDCP racemic mixture. The solution contained also EDTA [5 mM] or Ca^{2+} [2.5 mM] or Cu^{2+} [1 µM to 3 mM], or another metallic cofactor [100 µM]. The reaction was stopped by adding 40 µL of 0.2 M HCl. The released residual HDCP was removed by liquid-liquid extraction with 2 mL of 1,2-dichloroethane. Afterward this extract was centrifuged at 1000 g for 15 min. The extraction yield was over 98% in all cases. Fifteen µL of the organic solvent extract were finally injected into the HPLC system with an OA-4100 Techocel chiral column (HPLC Technology, Macclesfield, UK).

3. Results

3.1. Study strategy

To identify which protein(s) could be responsible for Cu^{2+}-enhanced hydrolysis, three commercially available proteins were studied that bind Cu^{2+}; a) human serum ceruloplasmine because it is responsible for 60% of Cu^{2+} transport in the organism; b) horse kidney metallothionein as it presents a high affinity to divalent cations, including Cu^{2+}, and plays a key role in the detoxification of xenobiotic agents in biological systems; c) chicken serum albumin (CSA) as it is responsible for 10% of Cu^{2+} transport in mammal blood, and also for its previously reported esterase activity. Their HDCP hydrolysis capacity was evaluated by the chiral chromatography method described in Materials and Methods. The total protein concentration was previously measured as 10 µL of serum by the Bradford method, and the albumin concentration was estimated by taking it to be 60% of serum proteins. In the tests, either 10 µL of chicken serum or 216 µg of CSA in 10 µL of buffer were used.

3.2. HDP enantiomers hydrolysis by ceruloplasmine and metallothionein

We observed that neither ceruloplasmine nor metallothionein presented the hydrolysis of any HDCP isomers at a Cu^{2+} concentration up to 100 µM (Figs. 1 and 2). At higher Cu^{2+} concentrations (up to 2.5 mM), some non-stereospecific hydrolysis occurred, mainly due to Cu^{2+} itself, as the effect in the controls with no protein demonstrated.

3.3. HDCP enantiomers hydrolysis by chicken albumin

Ten µL of chicken serum or 10 µL of deionized water containing 216 µg of CSA (the albumin content in 10 µL of serum) were incubated in 1 mL containing 400 µM of HDCP for 60 or 120 min, and the residual concentration of R-(+)-HDCP and S-(−)-HDCP was measured.

A significantly stereospecific hydrolysis took place R-(+)-HDCP > S-(−)-HDCP; 4:1), which was activated by 100 µM of Cu^{2+} in serum and with CSA at 60 and 120 min. Low non stereoselective HDCPase activity with EDTA was noted in both samples (Fig. 3). The activities for both biological samples were similar for the two experimental times. This experiment evidenced that albumin was the protein responsible for the Cu^{2+}-dependent stereoselective activity of chicken serum.

This selective R-(+)-HDCP hydrolysis was confirmed by incubating 216 µg/mL aliquots of CSA with different concentrations of Cu^{2+} (from 1 µM to 3 mM; Fig. 4), which led to the concentration that produced > 50% of maximum activation being around 60–100 µM, which

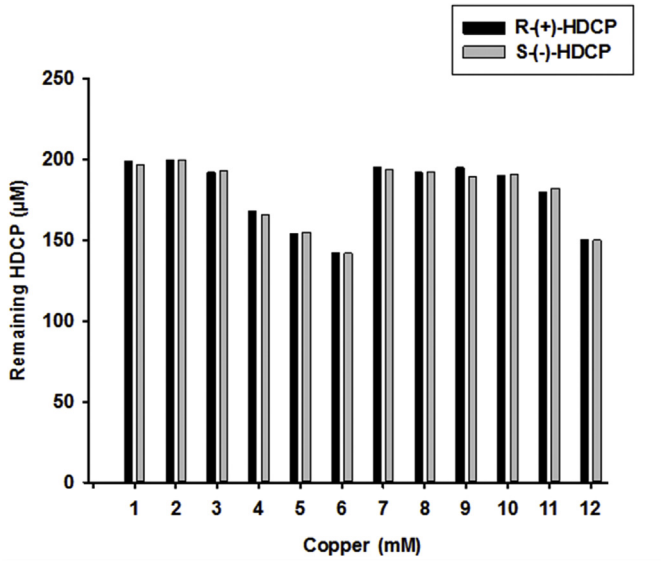

Fig. 1. Effect of copper on HDCP hydrolysis by ceruloplasmine. Values represent the mean of the three experiments run with 200 μg/mL of ceruloplasmin incubated with 400 μM of racemic HDCP (200 μM of each isomer) and the different Cu concentrations at 37 °C and pH 7.4 for 60 min. The SD was below 3%. Ceruloplasmine with 0 (1), 0.001 (2), 0.010 (3), 0.1 (4), 1 (5), 2 (6), and 2.5 (7) mM copper; (8) 2.5 mM EDTA; (9) 2.5 mM DTPA; Controls without ceruloplasmine with 0.1 mM (10), 1 mM (11) and 2.5 mM (12) of copper.

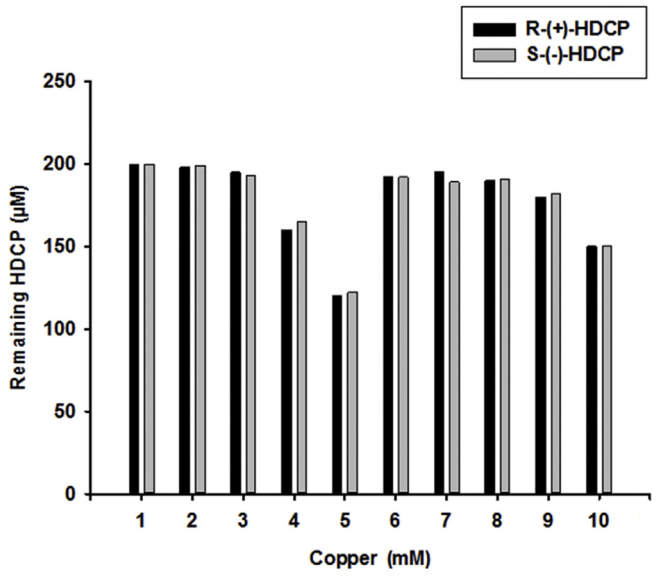

Fig. 2. Effect of copper on HDCP hydrolysis in metallothionein. Values represent the mean of the three experiments run with 200 μg/mL of metallothionein incubated with 400 μM of racemic HDCP (200 μM of each isomer) and the different Cu concentrations at 37 °C and pH 7.4 for 60 min. The SD was below 3%. Metallothionein with 0 (1), 0.010 (2), 0.1 (3), 1 (4), 2.5 (5) mM copper; 2,5 mM EDTA (6); 2.5 mM DTPA (7); Controls without metallothionein, with 0.1 mM (8), 1 mM (9) and 2.5 mM (10) copper.

is similar to that observed previously with chicken serum (Monroy-Noyola et al., 2017). Note that the isomers hydrolyses included the spontaneous hydrolysis caused by Cu^{2+} which, according to Fig. 1 and 2.5 mM of Cu^{2+} explains the S-(−)-HDCP hydrolysis. Thus, we can deduce that no enzymatic hydrolysis occurred with this enantiomer.

3.4. Selectivity of the Cu^{2+} effect as opposed to other divalent cations

Fig. 5 confirms the capacity to activate this "antagonistic

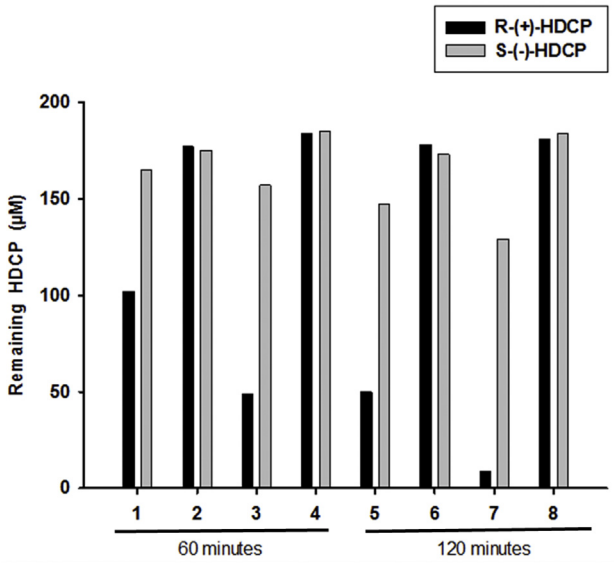

Fig. 3. Effect of copper on the stereospecific HDCP hydrolysis in serum and chicken albumin. Values represent the mean of the three experiments run with either 10 μL of serum or 10 μL of buffer containing 216 μg of CSA incubated with 1 mL of 400 μM of racemic HDCP (200 μM for each stereoisomer) in the presence of either 100 μM of copper or EDTA 5 mM at 37 °C and pH 7.4 for 60 or 120 min. The SD was below 1%. (1.5) Serum + Cu; (2.6) Serum + EDTA; (3.7) CSA + Cu; (4.8) CSA + EDTA.

stereoselectivity" as the hydrolysis in chicken albumin was specifically caused by Cu^{2+} because incubating this protein with 400 μM of HDCP in the presence of other divalent metals led neither to the hydrolysis of the racemic OP compound, nor to stereoselectivity.

4. Discussion

The Cu^{2+}-dependent HDCP hydrolysis results in this *ex vivo* study done by chiral chromatography using different commercial cuproproteins (ceruloplasmine, metallothionein and albumin) reveals that chicken serum albumin is the protein responsible for stereospecific Cu^{2+}-dependent R-(+)-HDCP hydrolysis.

The comparative levels of hydrolysis between 10 μL of chicken serum *versus* 216 μg of chicken serum albumin shows that it is very similar in terms of the level of hydrolysis and in terms of its stereoselectivity too (Fig. 3). The amount of albumin used in this experiment is the 60% of the total proteins in 10 μL the chicken serum as has been reported in the vertebrate sera. We cannot discard that other proteins might have some minor contribution, but albumin is able to account and interpreting quantitatively for around 100% of the cupper dependent activity observed in serum. This stereoselective hydrolysis copper-dependent of R-(+)-HDCP by chicken serum albumin was confirmed with different concentration of copper (Fig. 4) and not by others divalent cations (Fig. 5).

This is also known as "antagonistic stereoselectivity" (Monroy-Noyola et al., 2017), as it hydrolyzes more the enantiomer, which is less hydrolyzed in liver through Ca^{2+}-dependent activity (Monroy-Noyola et al., 1999a:1999b:2007). Of the three assayed cuproproteins, albumin is the protein that hydrolyzes in the same proportion as the HDCP hydrolysis level, and with the same stereoselectivity compared with serum. The activator effect of Cu^{2+} (100 μM) on the HDCP hydrolysis observed in serum and chicken albumin (>50%) is based on albumin's high affinity to the Cu^{2+} cation. The physiological role of 10% of the transport of this metal ion, which is essential for blood flow reaching vertebrate animal tissues, is attributed to this protein (Predki et al., 1992). Biochemical and biophysical studies have evidenced that bovine serum albumin presents specific metal binding sites, including Cu^{2+}, N-

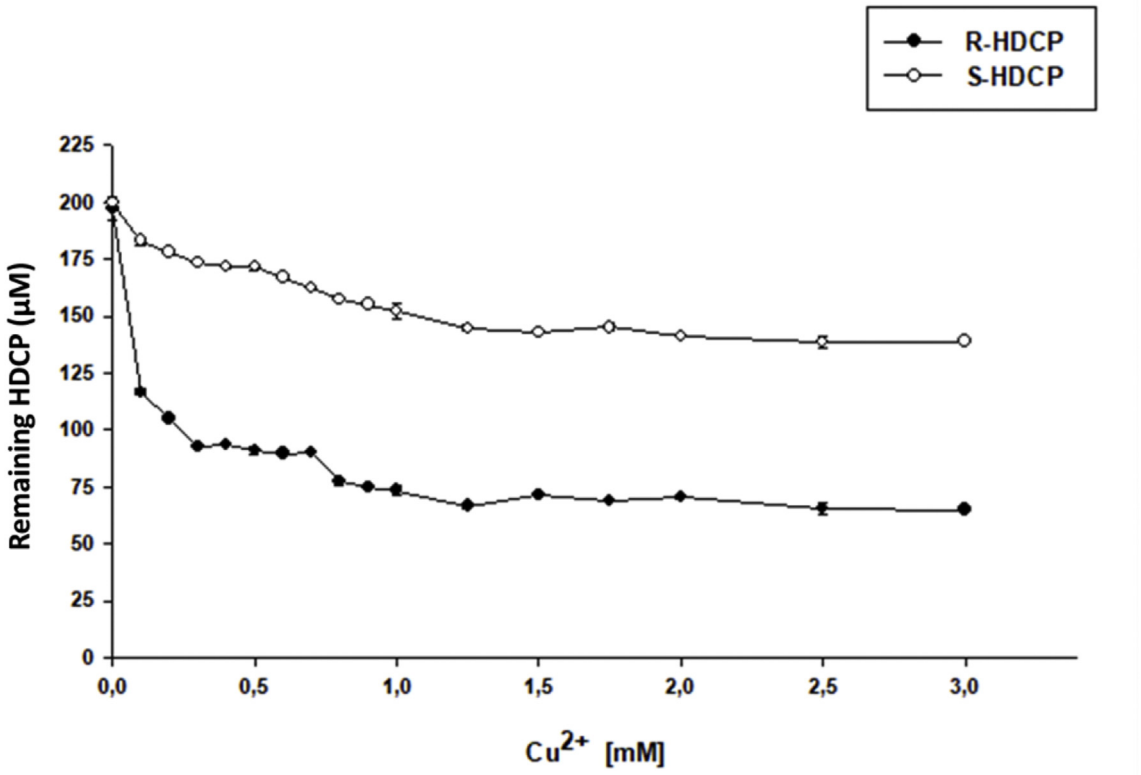

Fig. 4. Effect of the Cu concentration on the HDCP isomer hydrolysis in chicken albumin. Values represent the mean ± SD of the three experiments done with 216 µg/mL of CSA incubated with 400 µM racemic HDCP (200 µM for each isomer) and the different copper concentrations (between 0.1 and 3 mM) at 37 °C and pH 7.4 for 30 min.

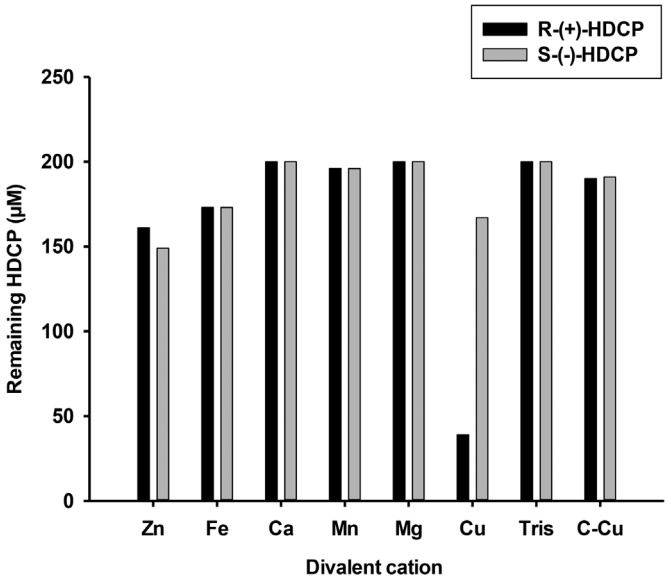

Fig. 5. Effect of the different divalent cations on the HDCP isomer hydrolysis in chicken albumin. Values represent the mean of the three experiments run with 216 µg/mL of CSA incubated with 400 µM racemic HDCP and 100 µM of metal cations or calcium 2.5 mM at 37 °C and pH 7.4 for 60 min. A copper control is included (100 µM without CSA (C-Cu). The SD was below 1%.

terminal site, Cys-34, multimetal-binding site (MBS) and another unknown site. This protein has been considered a model to study the binding of physiological transition metal ions in serum albumin in humans and other animals (Bal et al., 2013). Biochemical and biophysical studies suggest that the Cu^{2+} cation is nonspecifically bound to the N-terminal site, constituted by tetrapeptide Asp-Ala-Glu-His (Predki et al., 1992) of chicken serum albumin under similar pH and temperature physiological conditions to those employed in the HDCP hydrolysis experiments presented herein.

The esterase capacity of animal serum albumin in the absence of metal cofactors and physiological pH has been observed by Dirks and Boyer (1951), who reported the catalytic property of p-nitrophenyl esters using human serum albumin by identifying Tyr 411 as part of the catalytic center for the first time. This catalytic effect of albumin has been ratified by Kurono et al. (1992) and by our group (Sogorb et al., 1999) using OP compounds: 2,4-dinitro-phenyl diethylphosphate and HDCP, respectively. A rapid first Tyr 411 phosphorylation stage and a second slow dephosphorylation stage of this amino acid have been proposed as catalytic OPs hydrolysis (Sogorb et al., 1998). The capacity of albumin with no cofactor for hydrolyzing carbaryl has been reported (Sogorb et al., 2004a, 2004b). Moreover, at the relevant protein concentration *in vivo*, it has been demonstrated that albumin should be responsible for most detoxication by the hydrolysis of both carbaryl (Sogorb et al., 2007) and paraoxon, the parathion metabolite (Sogorb et al., 2008). In recent years, biochemical studies have reinforced phosphorylation knowledge about different tyrosine residues in albumin using commercial insecticides and nerve agents (Harald et al., 2010).

The present study reports the metal-dependent hydrolysis of an OP compound (HDCP) by chicken serum albumin (A-esterase activity) for the first time. This Cu2+-dependent activity is roughly 20-fold greater compared with its activity when metals are absent (incubation with 5 mM of EDTA) or in the presence of 2.5 mM of calcium (Monroy-Noyola et al., 2017). This suggests the participation of the N-terminal sequence or the multi-metal binding site (MBS) of the protein as the

catalytic centers of Cu2+-dependent R (+)-HDCP hydrolysis. This suggestion is made because Predki et al. (1992) reported a first Cu2+-binding site in this chicken plasma protein, whereas Bal et al. (1998) identified the MBS site as a second metal-binding site in albumins from other animals.

In conclusion, the present study evidences the A-esterase enzymatic property of albumin, and the fact that it is the protein responsible for R-(+)-HDCP Cu2+-dependent hydrolysis, which was observed in chicken serum ("antagonistic stereoselectivity") and was previously reported by our group (Monroy-Noyola et al., 2017).

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Transparency document

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