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Serum albumins and detoxication of anti-cholinesterase agents☆

Miguel A. Sogorb*, Eugenio Vilanova

Instituto de Bioingeniería, Unidad de Toxicología y Seguridad Química, Universidad Miguel Hernández de Elche, Spain

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ABSTRACT

Serum albumin displays an esterase activity that is capable of hydrolysing the anti-cholinesterase compounds carbaryl, paraoxon, chlorpyrifos-oxon, diazoxon and O-hexyl, O-2,5-dichlorphenyl phosphoramidate. The detoxication of all these anti-cholinesterase compounds takes place at significant rates with substrate concentrations in the same order of magnitude as expected during *in vivo* exposures, even when these substrate concentrations are between 15 and 1300 times lower than the recorded K_m constants. Our data suggest that the efficacy of this detoxication system is based on the high concentration of albumin in plasma (and in the rest of the body), and not on the catalytic efficacy itself, which is low for albumin. We conclude the need for a structure–activity relationship study into the albumin-associated esterase activities because this protein is universally present in vertebrates and could compensate for reduced levels of other esterases, i.e., lipoprotein paraoxonase, in some species. It is also remarkable that the biotransformation of xenobiotics can be reliably studied *in vitro*, although conditions as similar as possible to *in vivo* situations are necessary.

1. Introduction

Organophosphorus compounds (OPs)[1] and carbamates are mainly used as pesticides. OPs are chiefly used as insecticides, while carbamates are also used as fungicides, nematocides and herbicides [1,2]. Both types of compounds exert their pesticides effect through the inhibition of acetylcholinesterase (AChE) by either phosphorylation [1] or carbamylation [2] of a serine residue of the active centre of the enzyme. The study of the pesticide routes of biotransformation and detoxication is needed to perform appropriate risk assessments. In this sense, it is well-known that the main enzymes involved in the detoxication of OPs and carbamates are phosphotriesterases and carboxylesterases [3,4].

The most studied phosphotriesterase is paraoxonase (EC 3.1.8.1), which is a lipoprotein that is capable of hydrolysing paraoxon and other OPs, such as diazoxon or chlorpyrifos-oxon, in a calcium-dependent way [5]. Paraoxonase plays a critical role in the *in vivo* detoxication of certain OPs, such as diazoxon and chlorpyrifos-oxon [6–7], but surprisingly not in the detoxication of paraoxon since paraoxonase knock-out mice are not more sensitive to the toxic effects of paraoxon than wild-type animals [7]. This raises the question about the real role of paraoxonase in the detoxication of paraoxon.

Carboxylesterases are widely distributed enzymes in certain tissues, especially in the liver, brain and gastrointestinal tract [8]. These carboxylesterases can be stoichiometrically phosphorylated or carbamylated by OPs and carbamates, respectively, without detectable adverse effects. These reactions between non-essential carboxylesterases and OPs and carbamates are indeed a detoxication route since each scavenged molecule of pesticide is not available to reach the nervous system and to cause the toxic effects by inhibiting AChE [4].

Serum albumin displays an EDTA-resistant esterase activity which is capable of hydrolysing certain anti-cholinesterase compounds such as the OPs paraoxon, O-hexyl, O-2,5-dichlorophenyl phosphoramidate (HDCP) [9,10] and soman [11], and the carbamate carbaryl [12,13]. The interactions between OPs and albumin have recently drawn attention since the phosphorylated adducts of albumin might be used as biomarkers of exposure to OPs [14,15].

The enzymes involved in the detoxication of xenobiotics are usually studied *in vitro* under conditions that are far from the toxicologically relevant conditions *in vivo*. For human paraoxonase, the enzyme hydrolyses paraoxon, chlorpyrifos-oxon or diazoxon with K_m ranging (depending on the isoform) between 0.5 and 3 mM

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* Corresponding author at: Instituto de Bioingeniería, Unidad de Toxicología y Seguridad Química, Universidad Miguel Hernández de Elche; Avenida de la Universidad s/n, Alicante, 03202 Elche, Spain. Tel.: +34 966658506; fax: +34 965222033.
E-mail address: msogorb@umh.es (M.A. Sogorb).

[1] Abbreviations and organophosphorus compound common names: AChE, acetylcholinesterase; BSA, bovine serum albumin; BuChE, butyryl-cholinesterase; carbaryl, 1-Naphthalenylmethyl carbamate; chlorpyrifos, O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) thiophosphate; chlorpyrifos-oxon, O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphate; diazoxon, phosphoric acid, diethyl 6-methyl-2-(1-methylethyl)-4-pyrimidinyl ester; HDCP, O-hexyl, O-2,5-dichlorphenyl phosphoramidate; HSA, human serum albumin; OPs, organophosphorus compounds; paraoxon, O,O-diethyl 4-nitrophenyl phosphate.

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[7]. Thus, the hydrolysing activities associated with paraoxonase are studied *in vitro* using substrate concentrations of mM units, while the internal OPs concentrations during *in vivo* poisonings are several orders of magnitude lower than these figures. A similar conclusion may be drawn when we compare enzyme concentrations because the activities are assayed *in vitro* using highly diluted samples (a few μl of plasma/ml solution), while detoxication under physiological conditions takes place in 100% plasma.

The enzyme concentration may also prove a critical point to consider in order to gain understanding of the role of albumin in the detoxication of OPs and carbamate pesticides. The paraoxonase concentration in human plasma is around 50 mg/l [16] (approximately 1.3 μM when considering a molecular weight of 40 kDa [17]), while the albumin concentration is around 46 g/l [18] (670 μM when considering a molecular weight of 69 kDa [17]). This means that, *in vivo*, paraoxonase and OPs react at concentrations in

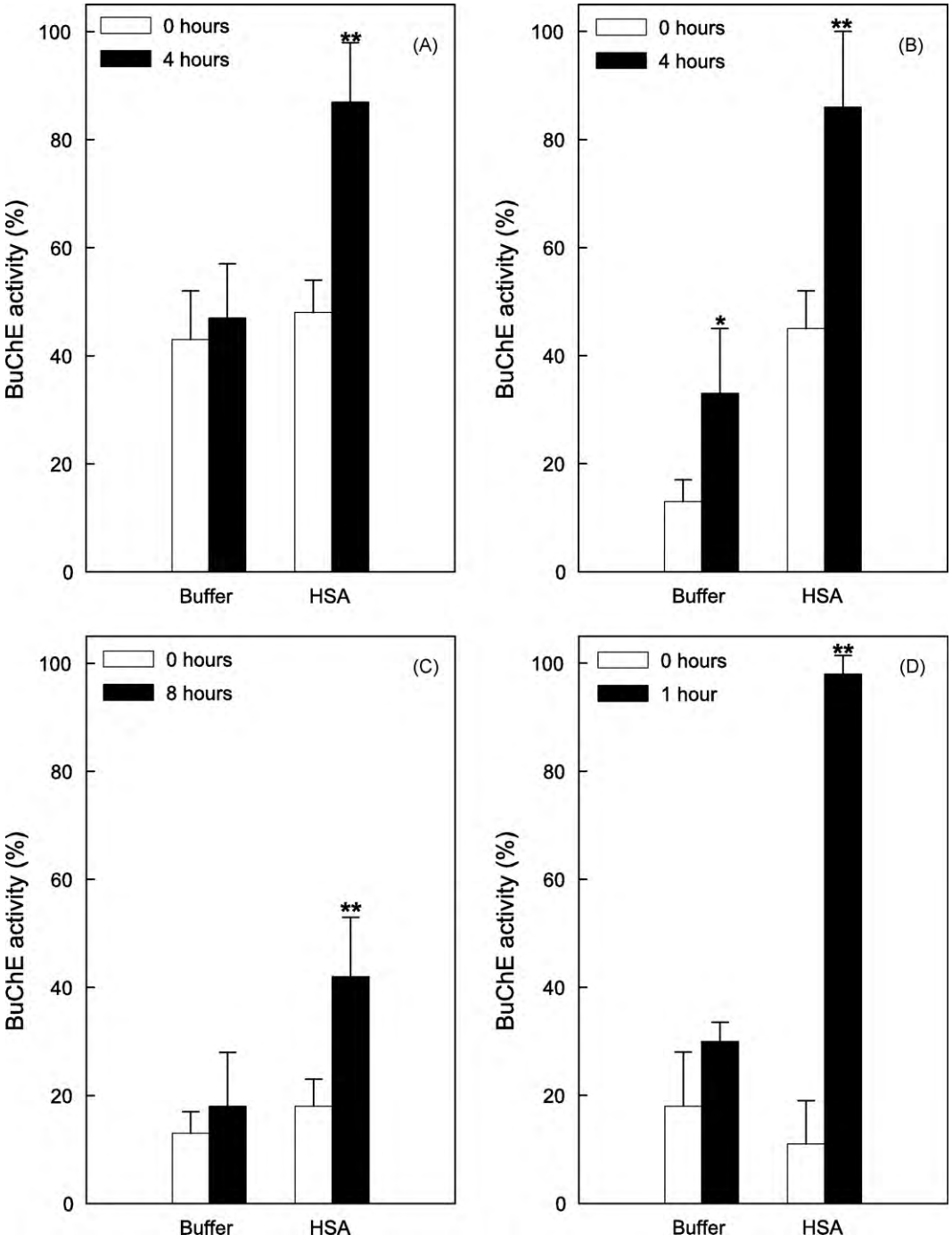

Fig. 1. Detoxication of OPs by human serum albumin. The hydrolyses of paraoxon (panel A), HDCP (panel B), diazoxon (panel C) and chlorpyrifos-oxon (panel D) were assayed by incubating solutions of 1 μM paraoxon, 20 μM HDCP, 80 μM diazoxon and 2 μM chlorpyrifos-oxon with 9 mg HSA/ml (131 μM HSA) (for paraoxon and HDCP) or 13 mg HSA/ml (189 μM HSA) (for diazoxon and chlorpyrifos-oxon), or with buffer (50 mM TRIS pH 7.4). After degradation, the inhibitory power against chicken serum BuChE of the resulting solutions was assayed. For this purpose, samples were incubated for 3 min at 37 °C with chicken serum, and the residual BuChE was assayed according to Ellman's procedure [27] and expressed as a percentage against a sample assayed in the absence of OP. The differences in the percentages of BuChE activity in the presence and absence of HSA without incubation (0 h of exposure) can be explained on the basis of the OPs quickly bound to albumin without turnover. In all cases, the mean ± SD of three independent samples is provided (*statistically different from the equivalent sample incubated for 0 h, $p < 0.05$; **statistically different from the equivalent sample incubated for 0 h, $p < 0.01$).

the same order of magnitude, while the albumin concentration *in vivo* is several orders of magnitude higher than their substrates.

In this work we compare the role of serum albumins in the detoxication of paraoxon, chlorpyrifos-oxon, diazoxon, HDCP and carbaryl under conditions that approach physiologically relevant conditions. For this purpose, we employed enzyme concentrations higher than the substrate, and substrate concentrations of the same order of magnitude as expected during moderate or acute poisoning. We concluded that serum albumin plays a critical role in the detoxication of paraoxon and carbaryl, and probably in that of HDCP, and is of minor (although not negligible) relevance in the detoxication of chlorpyrifos-oxon and diazoxon.

2. Materials and methods

2.1. Chemicals

Paraoxon, chlorpyrifos-oxon and diazoxon, with purities ranging between 94.5% and 96.0%, were obtained from Sigma Chemicals (Madrid, Spain), Dr. Ehrenstorfer (Augsburg, Germany), and Chem Service (West Chester, USA), respectively. HDCP (95% purity) was supplied by Dr. Nauman (Bayer Chemical Co., Germany). All other chemicals (always of analytical grade) were obtained from either Sigma Chemicals (Madrid, Spain) or local suppliers.

2.2. Biological materials

Human serum albumin (HSA) and bovine serum albumin (BSA), both essentially fatty acid-free and with 99% purity, were all obtained from Sigma Chemicals (Madrid, Spain). Chicken serum was also purchased from Sigma Chemicals.

2.3. Hydrolysis of paraoxon, chlorpyrifos-oxon, HDCP and diazoxon

The hydrolysis of paraoxon, chlorpyrifos-oxon, HDCP and diazoxon was mainly assayed by the loss of the inhibitory potency against chicken serum butyrylcholinesterase (BuChE) as a biosensor of OP degradation. For this purpose, the BuChE inhibitory potency of the solutions before and after incubation with HSA was compared. If incubation with HSA significantly lowered the OP concentration, then the solution's capability to inhibit the enzyme would also be lower. Similar approaches have been previously used successfully by other authors [19]. Chicken serum was chosen as a source of BuChE since it is well-known that this tissue does not express significant enzymatic activities involved in the hydrolysis of OPs [3]. In addition, the exposure time of BuChE to the tested solution was 3 min, a very short time compared with that employed for degradation (between 1 and 6 h, depending on the assayed OP).

In some cases (for determining kinetic constants), the hydrolysis of paraoxon, chlorpyrifos-oxon and HDCP was assayed using well-established spectrometric procedures based on monitoring the released hydrolysis product [9,20].

The hydrolysis of carbaryl was assayed by monitoring the 1-naphthol released during the carbaryl hydrolysis by gas chromatography coupled with mass spectrometry [21].

3. Results

3.1. Hydrolysis of organophosphorus compounds by human serum albumin

A 4 h incubation of 1 μM paraoxon with buffer at 37 °C brought no significant changes in the BuChE inhibition power of the solution (Fig. 1A). However, the residual BuChE inhibitory power after the incubation of the same 1 μM paraoxon solution with 9 mg HSA/ml (131 μM HSA) was 39% lower than before incubation (Fig. 1A).

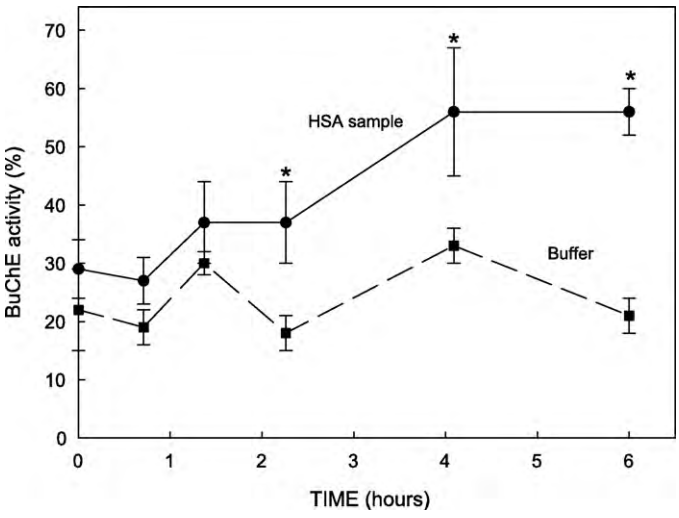

Fig. 2. Time course of paraoxon hydrolysis by human serum albumin. A solution of 0.33 μM paraoxon was incubated at 37 °C in the presence of either buffer or 9.5 mg HSA/ml (138 μM HSA). After incubation, an aliquot of each sample was incubated for 3 min at 37 °C with chicken serum. The residual BuChE was assayed according to Ref. [27], and expressed as, a percentage against a sample assayed in the absence of OP. In all cases, the mean ± SD of three independent samples is provided (*statistically different from the equivalent sample incubated with buffer for at least $p < 0.05$ or lower).

A 4 h incubation of a 20 μM HDCP solution with buffer or HSA at 37 °C led to significant reductions in the BuChE inhibitory potency in both cases, although this reduction was greater in the sample incubated with HSA (41%) than in that incubated with buffer (20%) (Fig. 1B).

Incubation of 80 μM diazoxon and 2 μM chlorpyrifos-oxon solutions with buffer did not alter the residual BuChE inhibitory potency of these solutions, although incubation with HSA lowered the BuChE inhibition potency by 24% and 80% in the cases of diazoxon (Fig. 1C) and chlorpyrifos-oxon (Fig. 1D), respectively. It is remarkable that chlorpyrifos-oxon was totally detoxified since the solution displayed no capability to inhibit chicken serum BuChE after 1 h of exposure to HSA.

The hydrolysis of paraoxon by HSA was time-dependent. The incubation of a solution of 0.33 μM paraoxon with buffer maintained the BuChE inhibition power of the solution (at around 75%) with no significant changes (Fig. 2). However, the incubation of this same paraoxon solution with 9.5 mg HSA/ml (138 μM HSA) caused a time-dependent reduction in the BuChE inhibition power. This reduction was statistically significant after 2 h (Fig. 2). The total reduction in the BuChE inhibition power reached 27% after 6 h of incubation with HSA (Fig. 2).

In order to gain knowledge of the physiological relevance of albumin-associated esterase activities, we determined the kinetics for the hydrolysis of paraoxon and chlorpyrifos-oxon. HSA hydrolysed chlorpyrifos-oxon with a higher V_{max} and K_m than paraoxon (Table 1). The catalytic efficiency (V_{max}/K_m) of HSA for the hydrolysis of chlorpyrifos-oxon was almost 4 times higher than for the hydrolysis of paraoxon (Table 1).

3.2. Hydrolysis of carbaryl by bovine serum albumin

The hydrolysis of carbaryl by BSA exhibited two well differentiated behaviours. BSA (7 mg/ml, 102 μM) was able to hydrolyse more than 75% of the carbaryl present in the solutions whose concentrations were higher than 50 μM in 3 h (Fig. 3). However, the

Table 1
Kinetic constants for the hydrolysis of several substrates by albumin. The hydrolysis of paraoxon and chlorpyrifos-oxon was assayed with HSA by spectrometric methods, while the hydrolysis of carbaryl was assayed with BSA by gas chromatography coupled with mass spectrometry [21]. V_{max} and K_m were obtained using the Lineweaver–Burk equation. The turnover numbers (k_{cat}) were calculated using a molecular weight of 69 kDa for both albumins [17] and by assuming that only one active centre in each molecule of enzyme is involved in the hydrolysis of the substrates.

Enzyme	Substrate	k_{cat} (min^{-1})	K_m (μM)	Catalytic efficiency (k_{cat}/K_m) (min^{-1} M^{-1})
HSA	Paraoxon	1.5×10^{-3}	377	3.9
HSA	Chlorpyrifos-oxon	4.0×10^{-2}	2640	15
BSA	Carbaryl	9.9×10^{-3}	380	26

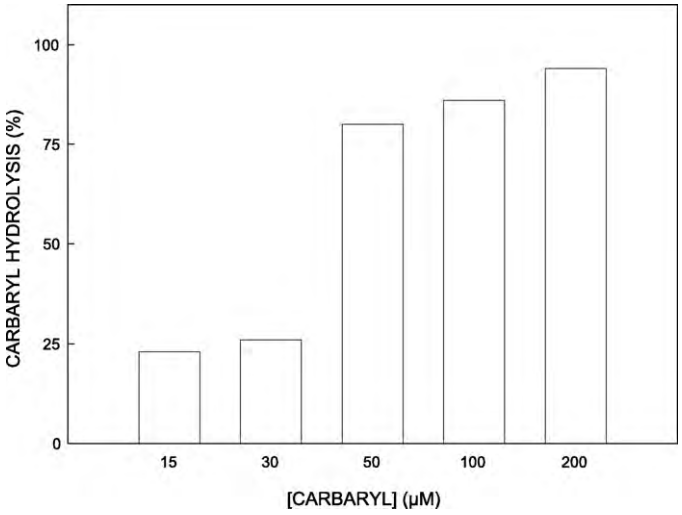

Fig. 3. Hydrolysis of carbaryl by bovine serum albumin. Carbaryl samples were incubated for 3 h with 7 mg HSA/ml (102 μM HSA) at 37 °C. The released 1-naphthol was extracted with an organic solvent and quantified by gas chromatography coupled to mass spectrometry [21], and was expressed as a percentage in relation to the initial carbaryl concentration.

total hydrolysed carbaryl was lower than 25% when the carbaryl concentration was 30 μM or lower (Fig. 3).

The kinetics for the hydrolysis of carbaryl by BSA showed a K_m in the same order of magnitude as the recorded K_m for the hydrolysis of paraoxon by HSA (Table 1), although the hydrolysis of carbaryl by BSA occurred with a V_{max} 8.5 times higher than the hydrolysis of paraoxon by HSA. The catalytic efficiency of BSA for the hydrolysis of carbaryl was 6.6 and 1.7 times higher than the catalytic efficiency of HSA for hydrolysing paraoxon and chlorpyrifos-oxon, respectively (Table 1).

4. Discussion

This work demonstrates that serum albumins are able to detoxify a variety of OPs as well as carbaryl. Hydrolysis takes place at significant rates at much lower substrate concentrations than, or in the same order of magnitude as, the recorded K_m. We consider that the most probable mechanism for detoxication of these substrates is its catalytic hydrolysis. Nevertheless, we cannot refuse the possibility of an alternative or simultaneous mechanism of detoxication in which certain side-chain of albumin might be phosphorylated or carbamylated without turnover.

4.1. Hydrolysing activities of organophosphorus compounds associated with human serum albumin under physiological conditions

In vitro assays for the biochemical and toxicological characterisation of biotransformation enzymes are usually performed under conditions that are far from physiologically relevant situations. We have attempted to study *in vitro* the hydrolysing activities in situations that mimic *in vivo* mild to severe poisonings.

There are no data available about the toxic concentration reached in the central nervous system during OP poisonings. Thus in each case, we chose the OP concentration that caused between 60% and 80% of the BuChE inhibition for 3 min of exposure. Under such conditions, HSA caused significant detoxication of paraoxon, HDCP, chlorpyrifos-oxon and diazoxon (Fig. 1) at concentrations of between 3.5 and 5.1 times lower than the physiological albumin concentration. This suggests that at 46 mg HSA/ml, that is, the real albumin concentration circulating in plasma [18], similar percentages of hydrolysis would be reached in one-third or one-fifth of the time employed for the assay.

Knocked-out mice for the paraoxonase gene were more susceptible to the toxic effect of chlorpyrifos-oxon than wild-type mice [6], although the sensitivity of these animals to paraoxon was similar to that of wild-type animals [7]. Li et al. [7] studied the catalytic efficiencies (V_{max}/K_m ratios) for the hydrolysis of paraoxon and chlorpyrifos-oxon by paraoxonase. They concluded that the isoenzyme R192 hydrolysed chlorpyrifos-oxon with an efficacy 41 times higher than paraoxon, while this record increased to 214 in the case of the isoenzyme Q192. These figures, together with the findings reported in this work, might explain the surprising results obtained with transgenic mice. Indeed, the K_m for paraoxon hydrolysis was lower than for chlorpyrifos-oxon hydrolysis (Table 1), thus enabling albumin to hydrolyse paraoxon more efficiently than chlorpyrifos-oxon at the lower substrate concentrations found during *in vivo* exposures. In conclusion: (1) differences in the catalytic efficiencies determine that the absence of paraoxonase is more relevant for chlorpyrifos-oxon detoxication than for paraoxon detoxication and (2) differences in the K_m determine that transgenic animals can overcome the lack of paraoxonase with albumin for the detoxication of paraoxon, but not for the detoxication of chlorpyrifos-oxon. Previous studies in which we studied the capability of human serum to detoxify paraoxon and chlorpyrifos-oxon by calcium-dependant and EDTA-resistant routes corroborate these conclusions [22].

4.2. Hydrolysing activities of carbaryl associated with human serum albumin under physiological conditions

As with OPs, there is no information available about the internal carbaryl concentration reached during carbaryl poisonings. Gordon and co-workers [23] orally dosed rats with 175 mg carbaryl/kg bw to cause around 50% of brain cholinesterase inhibition 4 h after exposure. It is well-established that carbaryl is totally absorbed by the gastrointestinal route and does not accumulate in the organism [24]. Therefore, this dose might cause an internal exposure of 870 μM, which is around twofold higher than the recorded K_m (Table 1). By extrapolating the data shown in Fig. 3, we will be able to estimate that this carbaryl could be hydrolysed after a few hours of exposure to 46 mg HSA/ml.

Li et al. [16] reported the presence of BuChE, paraoxonase and albumin esterase, but not carboxylesterase, in human plasma.

Paraoxonase is unable to hydrolyse carbaryl, thus the detoxication of this compound must necessarily be assigned to either albumin or BuChE. Albumin concentration is around 46 mg/ml (670 μM) while BuChE concentration is 5 mg/l (60 nM with a molecular weight of 85 kDa). Therefore if we consider the above estimated internal carbaryl concentrations, we may conclude that most of the carbaryl will be detoxified by albumin. In mouse plasma, a third esterase (a carboxylesterase), which is capable of hydrolysing carbaryl, can be found at 80 mg/l [16] (1.3 μM with a molecular weight of 62 kDa [17]). Therefore, this carboxylesterase might contribute to carbaryl detoxication to a greater extent than BuChE, although the main relevance must still be assigned to albumin. For all these enzymes (albumin, BuChE and carboxylesterase), the carbaryl detoxication mechanism may include a spontaneous reactivation of the carbamylated active centres. Indeed, it is well-known that the carbamylation of Ser residues (and probably that of the Tyr residues as well) is not as stable as phosphorylation because the carbamylated enzyme can be reactivated by nucleophilic molecules (usually water molecules) [4].

4.3. Final remarks and conclusions

Our data suggest that albumin plays a highly relevant role in the detoxication of paraoxon and carbaryl (and probably that of HDCP as well), but plays a minor role in the detoxication of chlorpyrifos-oxon and diazoxon. The capability of serum albumin to hydrolyse paraoxon and carbaryl has long since been known [25,26]. However, the role of these hydrolysing activities has been underestimated given the low catalytic efficacy of albumin, especially when compared with other enzymes such as paraoxonase. Yet low catalytic efficiency is compensated by the extremely high enzyme concentration (46 mg/ml, 670 μM), similarly to insect strains that over-express non-essential carboxylesterases for detoxifying OPs and carbamates for developing resistances [4]. The traditional underestimation of the role of albumin in the detoxication of paraoxon has been due to the performance of *in vitro* assays in conditions far from physiological situations, and the subsequent extrapolation of the conclusions to *in vivo* exposures.

This work demonstrates that: (1) biotransformation (activation and detoxication) might occur by unexpected routes and (2) the biotransformation of xenobiotics can be studied *in vitro*, although it is mandatory to use conditions that mimic the *in vivo* situations as much as possible.

Finally, we conclude the need for a structure–activity relationship study into the esterase activities associated with albumin because this protein is universally present in vertebrates and could confer resistance to those individuals or species with reduced levels of other esterases.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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