Rabbit Serum Albumin Hydrolyzes the Carbamate Carbaryl

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One of the main detoxification processes of the carbamate insecticides is the hydrolysis of the carbamic ester bond. Carboxylesterases seem to play important roles in the metabolization of carbamates. This study performs a biochemical characterization of the capabilities of rabbit serum albumin (RSA) to hydrolyze the carbamate carbaryl. Rabbit serum albumin was able to hydrolyze carbaryl with a K_{cat} of 7.1×10^{-5} s^{-1}. The K_m for this hydrolysis reaction was 240 μM. Human, chicken, and bovine serum albumins were also able to hydrolyze carbaryl. The divalent cation Cu^{2+} at 1 mM concentration inhibited around 50% of the hydrolysis of carbaryl by RSA. Other mono- and divalent cations at 1 mM concentration and 5 mM EDTA exerted no significant effects on the hydrolysis of carbaryl by RSA. The inhibition of the carbaryl hydrolysis by sulfydril blocking agents suggests that a cysteine residue plays an important role in the active center of the catalytic activity. Both caprylic and palmitic acids were noncompetitive inhibitors of the carbaryl hydrolysis by RSA. The carboxyl ester *p*-nitrophenyl butyrate is a substrate of RSA and competitively inhibited the hydrolysis of carbaryl by this protein, suggesting that the hydrolysis of carbaryl and the hydrolysis of carboxyl esters occur in the same catalytic site and through a similar mechanism. This mechanism might be based on the carbamylation of a tyrosine residue of the RSA. Serum albumin is a protein universally present in nontarget species of insecticides; therefore, the capability of this protein to hydrolyze other carbamates must be studied because it might have important toxicological and ecotoxicological implications.

Introduction

Chemical pesticides are actually widely used in agriculture because they contribute to pest control in a very effective way. The three main groups of chemical insecticides are (a) organophosphorus compounds, (b) pyrethroids, and (c) carbamates. Among all of these groups, carbamates probably own the widest range of pesticide activities because they are insecticides, fungicides, and herbicides. Carbamates are widely used insecticides in agriculture and in urban gardens. They are also effective in controlling pest in buildings and on a wide range of indoor plants (*1*).

The acute toxic effects of organophosphorus and carbamate pesticides are based on the inhibition of acetylcholinesterase. Indeed, they are able to phosphorylate or carbamylate a serine residue of acetylcholinesterase, yielding an inhibited enzyme and originating the typical cholinergic acute effects (lacrimation, salivation, miosis, convulsions, and death) on the basis of acetylcholine accumulation on synaptic terminals (*1*). However, while the phosphorylation of the enzyme is irreversible, the carbamylated enzyme−inhibitor complex can be hydrolyzed by a water molecule, yielding a free active enzyme (*1*).

In addition to the described cholinergic effects, the carbamates exhibit other important toxic effect called "promotion" (*2*). When animals are treated with carbamates after subneuropathic insults (organophosphates, 2,5-hexanedione, bromophenylacetylurea, or even traumatic axonopathy (*3*)), the subsequent neuropathy is promoted or becomes more severe.

The selective toxicity of chemical insecticides is based on the existence of effective detoxification enzymes in the nontarget species (essentially mammals and birds). Therefore, understanding the detoxification routes might be extremely important in the risk assessment of the carbamates and other chemical insecticides.

The two main routes for the detoxification of carbamates are the oxidation and the hydrolysis of the carbamic ester bond (*1*). The chemical structure of carbamate insecticide renders these compounds susceptible to hydrolysis by carboxylesterases. In addition, the metabolites resulting from the oxidation of parental carbamate still conserve the intact carbamic bond; therefore, they can be hydrolyzed by carboxylesterases. Thus, hydrolysis seems to be a critical step in the detoxification route of carbamates.

Several carboxylesterases with a capability to hydrolyze different carbamates have been studied in various bacteria strains, like *Blastobacter* sp. (*4*), *Arthrobacter* (*5*), *Pseudomonas* (*6*), *Achromobacter* sp. (*7*), and *Micrococus* sp. (*8*). However, to our knowledge, detailed toxicological and biochemical studies about carboxylesterases able to hydrolyze carbamates in vertebrates are not available.

Because carbaryl (1-naphthalenylmethyl carbamate) has a relatively simple structure, this compound is an

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appropriate model for toxicological and metabolic studies of carbamate insecticides (*1*). We have performed a partial characterization of the capability of rabbit serum albumin (RSA)[1] to hydrolyze carbaryl. Michaelis kinetic parameters for the hydrolysis of the substrate and for the inhibition of the hydrolysis by several compounds were studied. The response of the activity to EDTA and to several divalent cations was also characterized. All of the collected data allowed for the proposition of a hydrolysis mechanism on the basis of the carbamylation of a tyrosine residue of RSA.

Experimental Procedures

Biological Tissues. All biological tissues were supplied by Sigma-Aldrich Química S.A. (Madrid, Spain). The employed tissues were (a) rabbit serum albumin (RSA) (Sigma product A-9438), (b) chicken serum albumin (CSA) (Sigma product A-3686), (c) human serum albumin (HSA) (Sigma product A-1887), (d) bovine serum albumin (BSA) (Sigma product A-4503), and (e) rabbit serum (Sigma product R-4505). According to the supplier catalog, the purity of the albumin samples ranged from 96% to 99%, with RSA and HSA also being essentially fatty acid-free.

Chemicals. Caution: *The following chemicals are hazardous and should be handled carefully.* Carbaryl(1-naphthalenylmethyl carbamate) of 99.5% purity and permethrin ((3-phenoxyphenyl)methyl-3-(2,2-dichloroethenyl)-2,2-dimethyl cyclopropanecarboxylate) of 95.5% purity were supplied by Labor Dr Ehrenstorfer-Schäfers (Augsburg, Germany). *O*-hexyl-*O*-2,5-dichlorophenyl phosphoramidate (HDCP) (purity higher than 99%) was supplied by Dr Nauman (Bayer Chemical Co., Germany). The carboxyl ester *p*-nitrophenyl butyrate (*p*-NPB) and all other chemicals were supplied at analytical grade by Sigma-Aldrich Química S.A..

Enzymatic Assays. Carbaryl hydrolyzing activity (carbarylase) was assayed monitoring the release of hydrolysis product (1-naphthol) by a colorimetric method (*9*). Standard assays were performed as follows: 50 μL of 12 mM carbaryl in dried acetone was mixed with 500 μL of 200 mM Tris (pH 7.4). Tissue at the appropriate concentration was added to make a final volume of 1 mL. Typical standard assays were performed with 350 μL of serum or 3.5 mg of albumin. This mixture was incubated at 37 °C for 5 h. The reaction was stopped by adding 350 μL of 2% SDS/1.2 mM 4-aminoantipyrine in 50 mM Tris (pH 8.0). The complex formed by the released 1-naphthol and 4-aminoantipyrine was further reduced by 175 μL of 30 mM potassium hexacyanoferrate (III). The color of the reduced complex (ϵ = 7100 ± 600 M^{-1} cm^{-1}) was determined at 510 nm. The concentration of 1-naphthol was calculated by comparing the results with a calibration curve (range between 5 and 100 μM). All values were corrected for nonenzymatic hydrolysis with samples assayed in identical conditions without biological tissue.

Kinetics. For the determination of Michaelis constants, standard assays were performed employing several carbaryl concentrations in the range of 60−1200 μM. The results were fitted to the Lineweaver−Burk equation using SigmaPlot 5.0 software (Sigma-Aldrich Química S. A.).

Effect of Cations and Organic Compounds. Reaction samples were incubated as was described previously in the presence of the appropriate compound. The type of inhibition was determined by incubating several inhibitor concentrations in the presence of several substrate concentrations. Finally, the type of inhibition and the K_i was calculated fitting the results to the Lineweaver−Burk equation by using SigmaPlot software.

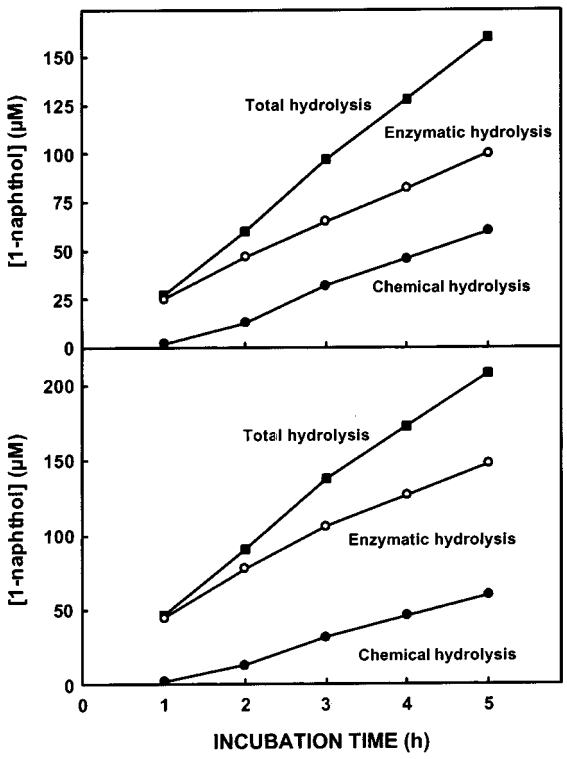

Figure 1. Time course of carbaryl hydrolysis by rabbit serum (top panel) and RSA (bottom panel). The concentration of 1-naphthol is plotted against the incubation time. The total hydrolysis (■) of carbaryl was assayed at 37 °C and pH 7.4 in the presence of 350 μL of rabbit serum (top panel) or 3.5 mg of RSA (bottom panel). Chemical hydrolysis (●) was monitored under identical conditions in absence of biological tissues. In all cases, the carbaryl concentration was 600 μM. Enzymatic hydrolysis (○) was calculated at each time as the difference between total hydrolysis and chemical hydrolysis.

Results

Hydrolysis of Carbaryl by Biological Tissues. When carbaryl was incubated with rabbit serum, the release of 1-naphthol was detected. The amount of released 1-naphthol was time-dependent and was linear from 1 to 5 h (Figure 1). The specific activity of rabbit serum carbaryl hydrolyzing activity (carbarylase) is displayed in Table 1.

The release of 1-naphthol is consistent with the hydrolysis of the carbamic ester bond of carbaryl or any of its oxidized metabolites. The capability of serum albumins to hydrolyze several carboxyl esters (*10*−*12*), phosphoric triesters (*13*), and phosphoramidates (*13*, *14*) was previously reported. Therefore, we decided to test the capability of RSA to hydrolyze carbaryl. RSA was also able to hydrolyze carbaryl in a time-dependent way (Figure 1). The specific carbarylase activity of RSA was 23 nmol of 1-naphthol/5 h/mg of protein (Table 1).

Chicken, human, and bovine serum albumin also displayed carbarylase activity (Table 1). However, among all of the assayed tissues, RSA exhibited the highest level of carbarylase activity; the lowest level was detected for CSA (Table 1).

To obtain in the reaction media enough concentration of carbaryl for estimating, with accuracy, the Michaelis kinetic constants, the acetone concentration was in-

[1] Abbreviations: BSA, bovine serum albumin; carbarylase, carbaryl hydrolyzing activity; CSA, chicken serum albumin; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); HDCP, *O*-hexyl-*O*-2,5-dichlorophenyl phosphoramidate; $Hg(OAc)_2$, phenylmercuric acetate; MVK, methyl vinyl ketone; *N*-EM, *N*-ethylmaleimide; *p*-OHMB, *p*-hydroxymercuribenzoic acid; *p*-NPB, *p*-nitrophenyl butyrate; RSA, rabbit serum albumin.

Table 1. Carbaryl Hydrolyzing Activities of Rabbit Serum and Serum Albumins from Various Species[a]

tissue	specific activity[b]	K_{cat} (s^{-1})	K_m (μM)
rabbit serum	1.5 ± 0.2 ($n = 12$)		
serum albumin of			
rabbit	23 ± 4 ($n = 17$)	$(7.1 \pm 1.4) \times 10^{-5}$ ($n = 4$)	240 ± 60 ($n = 4$)
chicken	4.4 ± 0.7 ($n = 3$)		
human	16 ± 3 ($n = 3$)		
bovine	13 ± 2 ($n = 3$)		

[a] In all cases, mean ± SD for n independent experiments is displayed. Specific activity was calculated using 600 μM substrate concentration. K_m and K_{cat} were obtained incubating different substrate concentration (over range of 60−1200 μM) with RSA at 37 °C during 5 h at pH 7.4. Michaelis kinetic parameters were calculated according to the Lineweaver−Burk equation. K_{cat} was calculated assuming a molecular weight of 64 300 for RSA, one unique active center in each molecule, and that the release of product is linear for 5 h of reaction (see Figure 1). [b] nmol of 1-naphthol/5 h/mg of protein.

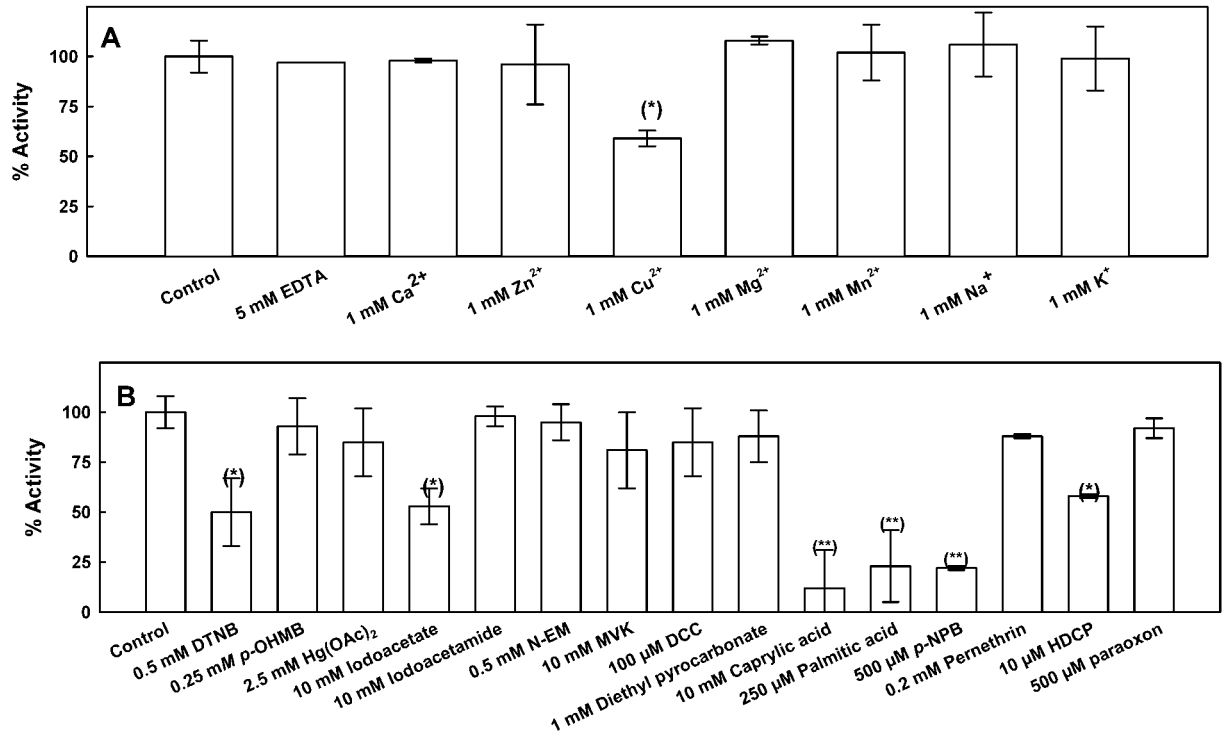

Figure 2. Effect of EDTA and different cations (panel A) and of different organic compounds (panel B) on hydrolysis of carbaryl by RSA. Activity was assayed incubating 3.5 mg of RSA with 600 μM substrate at pH 7.4 during 5 h at 37 °C in the presence of cations, EDTA, or the organic compound at the concentration displayed in each case. Plotted is the mean ± SD for at least three independent experiments. Control exhibited values similar to those displayed in Table 1: (*) statistically different from control, $p < 0.01$; (**) statistically different from control, $p < 0.001$). Abbreviations are as previously defined.

creased to 15%. Standard assays were performed with 15% acetone in the medium and several substrate concentrations. In these conditions, RSA hydrolyzed carbaryl with a K_{cat} of 7.1×10^{-5} s^{-1} and a K_m of 240 μM (Table 1).

Effect of EDTA and Metals on Carbaryl Hydrolyzing Activity of RSA. To find evidence if carbarylase activity of RSA is metal-dependent, the hydrolysis of carbaryl was assayed in standard conditions in the presence of 5 mM EDTA or 1 mM Ca^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Na^+, and K^+. Among all of the assayed metals, Cu^{2+} caused significant inhibition of carbarylase activity (Figure 2A). All other cations and EDTA caused no significant effect (neither inhibition nor activation) on carbarylase activity (Figure 2A).

Effect of Several Organic Compounds on Carbaryl Hydrolyzing Activity of RSA. To perform a biochemical characterization of the active center of carbarylase activity, the effects of several organic compounds (nine selective amino acid blocking chemicals, one short- and one long-chain fatty acid, one carboxyl ester; one pyrethroid, one phosphoramidate, and one phosphoric triester) were studied. Standard assays in the presence of those compounds at the concentrations indicated in Figure 2B were performed. Among all of the assayed compounds, DTNB and iodoacetate (cysteine-blocking reagents) and the phosphoramidate HDCP were able to inhibit carbarylase activity at around 50% (Figure 2B). Stronger inhibition was detected when caprylic, palmitic, and *p*-NPB were used as inhibitors (Figure 2B).

To study the type of inhibition by fatty acids, several substrate concentrations were incubated in the presence of several caprylic (from 0.1 to 10 mM) or palmitic (from 6 to 300 μM) acid concentrations. In these experiments, the acetone concentration in the medium was increased to 15% for identical reasons as those outlined previously. When V_{max} and K_m were calculated for each inhibitor concentration, we detected that K_m was maintained approximately constant while V_{max} decreased when the inhibitor concentration was increased. It suggested noncompetitive inhibition. The K_i constants were calculated using Lineweaver−Burk plots (Table 2). In the case of caprylic, a K_i of 480 μM was estimated. However, the K_i was around 3.7 times lower for the inhibition of palmitic

Table 2. Inhibitory Effect of Some Organic Compounds on Carbaryl Hydrolyzing Activity of RSA[a]

compound	K_i (μM)	type of inhibition
caprylic acid	480	noncompetitive
palmitic acid	130	noncompetitive
p-nitrophenyl butyrate	45	competitive

[a] Different substrate concentrations were incubated with different inhibitor concentrations at 37 °C during 5 h at pH 7.4. After that, color was developed as in standard protocol. Type of inhibition and K_i were determined according to the Lineweaver−Burk equation.

Table 3. Additive Inhibitory Effect of Cu^{2+} and *p*-Nitrophenyl Butyrate on Carbaryl Hydrolyzing Activity of RSA[a]

condition	activity[b]	% inhibition
control	19	0
50 μM *p*-nitrophenyl butyrate	11	42
0.5 mM Cu^{2+}	17	11
50 μM *p*-nitrophenyl butyrate + 0.5 mM Cu^{2+}	8.4	56

[a] Carbaryl hydrolyzing activity was assayed in standard conditions (3.5 mg of RSA, pH 7.4, 600 μM substrate, and 5 h of incubation at 37 °C) in absence of inhibitors (control) or in the presence of *p*-nitrophenyl butyrate, Cu^{2+}, for both compounds. [b] nmol of 1-naphthol/5 h/mg of protein.

acid (Table 2).

The carboxyl ester *p*-NPB displayed a strong inhibitory potency toward RSA carbarylase activity (Figure 2B). When kinetic parameters were calculated in the presence of 15% acetone and several *p*-NPB concentrations, we detected that V_{max} maintained constant. However, K_m increased with the inhibitor concentration. These data are consistent with competitive inhibition. The K_i displayed by *p*-NPB was lower than the K_i exhibited by fatty acids (Table 2).

Copper at a 1 mM concentration was able to inhibit the hydrolysis of carbaryl by RSA at around 50% (Figure 2). It is not possible to reach higher Cu^{2+} concentrations at pH 7.4; therefore, it was not possible to reach enough inhibition for a detailed analysis of the inhibition. However, we detected additional inhibitory effects of Cu^{2+} and *p*-NPB (Table 3), suggesting that the carboxyl ester and the cation exerted their respective actions in different sites of the enzyme.

Iodoacetate and DTNB displayed the capability to inhibit the carbarylase activity of RSA. However, because it was not possible to obtain high inhibition percentages at the maximum inhibitor concentration and because they are nonreversible blocking agents, it was not possible to perform a Michaelis analysis of the inhibition. We detected that both reagents inhibited carbarylase activity in a concentration-dependent way (Figure 3). The maximum inhibition percentage was, in both cases, around 50%. However, DTNB is the most powerful inhibitor because this maximum inhibition was reached at a lower concentration of DTNB than for iodoacetate.

Reactivation of RSA Carbarylase Activity Inhibited by HDCP. The phosphoramidate HDCP was previously described for CSA as an inhibitor of *p*-NPB hydrolyzing activity (*14*). Both substrates are hydrolyzed by CSA in the same active center by a similar mechanism (*14*). HDCP and *p*-NPB have also demonstrated the capability to inhibit the hydrolysis of carbaryl by RSA (Figure 2B). In addition, RSA is able to hydrolyze HDCP (*13*), carbaryl (Table 1), and *p*-NPB. All these data

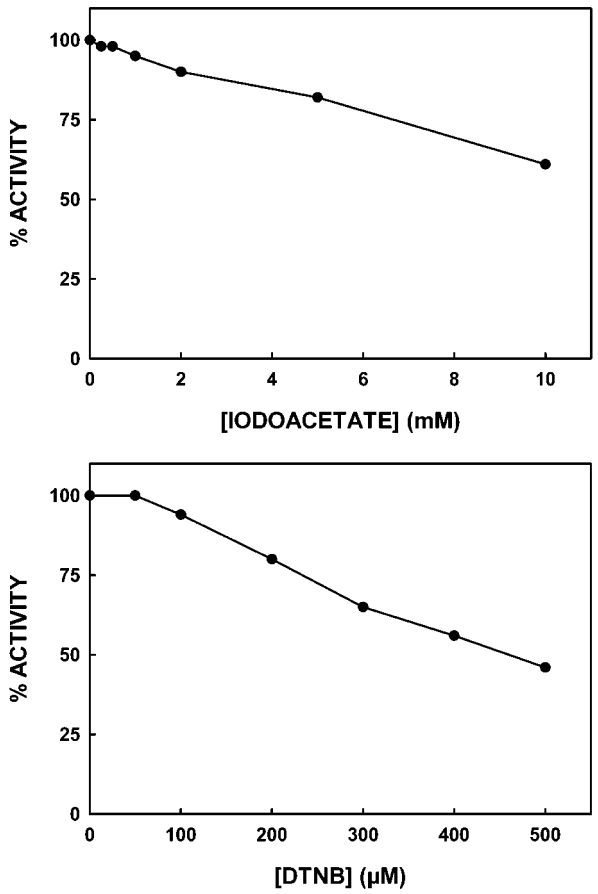

Figure 3. Effect of iodoacetate (panel A) and DTNB (panel B) on the hydrolysis of carbaryl by RSA. The effect of sulfydril blocking agents on carbaryl hydrolyzing activity was tested assaying the activity using 600 μM substrate, 5 h of incubation at 37 °C and pH 7.4, and different concentrations of iodoacetate and DTNB. Control exhibited values similar to those displayed in Table 1.

suggest that *p*-NPB, HDCP, and carbaryl might be hydrolyzed at the same active center.

The solubility of the phosphoramidate HDCP is extremely low. It does not allow for the study of the kinetics of the inhibition of carbarylase activity through standard analysis procedures. To determine if carbaryl and HDCP are hydrolyzed by RSA in the same active center, we incubated a mixture containing 160 μM RSA and 40 μM HDCP at 37 °C for 15 min. We established that 90% of HDCP present in the mixture had been hydrolyzed by RSA (data not shown) in these conditions. Afterward, the mixture was diluted 5 times, and the incubation at 37 °C was prolonged. Aliquots of the diluted mixture were extracted at different times, then carbaryl was added, and carbarylase activity was assayed according to the standard procedure. The results were compared with control samples initially incubated in absence of HDCP. Immediately after dilution, the sample incubated with HDCP displayed around 50% activity of the control (Figure 4). The percentage of activity was increased when the incubation at 37 °C was prolonged, reaching 90% of control activity for 2 h of incubation.

Discussion

This study is (to our knowledge) the first detailed biochemical report on the capability of RSA to hydrolyze the carbamate carbaryl.

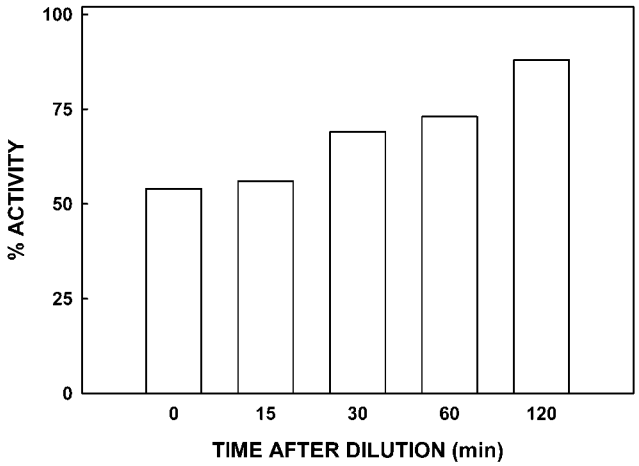

Figure 4. Reactivation of the carbaryl hydrolyzing activity in RSA after inhibition by HDCP. A mixture with 160 μM RSA plus 40 μM HDCP was incubated at 37 °C during 15 min. Afterward, the mixture was diluted 5 times, and the incubation at 37 °C was prolonged. Finally, carbaryl was added at different times, and carbaryl hydrolyzing activity was assayed in standard conditions. Results were compared with control samples treated in identical conditions in absence of HDCP. There were no significant differences among control samples incubated during different times. Controls exhibited values similar to displayed in Table 1.

The hydrolysis of carbaryl by RSA was inhibited by fatty acids in a noncompetitive way (Table 2), suggesting that fatty acids bind to the enzyme in a site not related to the active center. Fatty acids also have been described as inhibitors of several carboxylesterase (*10*−*11*, *14*) and phosphotriesterase activities (*14*) of serum albumins. It is well-known that albumin has at least six different binding sites for fatty acids, with three of them being unknown (*15*). We conclude that the fatty acid does not bind in the catalytic site involved in the carbaryl hydrolysis. The inhibition might be explained if we assume that the binding of fatty acids to RSA might alter the conformation of the enzyme, resulting in reduced hydrolytic activity.

Serum albumin accounts for around 50% of the total serum proteins (*16*). If we use these data to extrapolate the specific carbarylase activity of the RSA contained in our rabbit serum sample, we conclude that RSA present in serum displays around 8 times lower activity than the sample of purified RSA. The explanation for this phenomena can be attributed to inhibition by fatty acids. Fatty acids circulate in serum at a total concentration of just under 1 mM, and only less than 0.1% of them circulate in free form in plasma, with the rest being bound to albumin (*15*). According to that and to the data shown in Figure 2B, RSA present in rabbit serum must be inhibited by bound fatty acids. However, according to supplier information, the preparation of pure RSA is fatty acid-free; therefore, its activity is not inhibited by these acids.

EDTA had no significant effect on carbarylase activity, indicating that there are no metals in the active center of RSA (Figure 2A). This is supported by the observation that none of the tested cations activated the carbarylase activity. The phosphotriesterase activity found in RSA is also EDTA-resistant, while other phosphotriesterase activities found in rabbit serum lipoproteins fraction are Ca^{2+}-dependent and, therefore, EDTA-sensitive (*13*).

Copper and *p*-NPB exerted an inhibitory effect on RSA carbarylase activity (Figure 2). These effects seem to be additive (Table 3). It suggests that Cu^{2+} exerts its effects at a site other than the active center. It is previously reported that albumin posseses binding sites for Cu^{2+} (*15*). These data suggest that the binding of Cu^{2+} to RSA might alter the conformation of the active center, generating an enzyme molecule that is still able to hydrolyze carbaryl but with less efficacy than an enzyme without Cu^{2+}. This effect is in contrast with the activation by Cu^{2+} of the HDCP hydrolyzing activity found in CSA (*17*). It might be explained if we consider that the changes originating in the enzyme by the binding of Cu^{2+} originate slight modifications on the active center that make the enzyme more effective for the hydrolysis of HDCP but less effective for the hydrolysis of carbaryl.

Iodoacetate and DTNB are able to inhibit the hydrolysis of carbaryl by RSA (Figure 2B). However, the maximum inhibition is around 50% (Figure 4). This suggests that there are some cysteine residues in the protein that are necessary for the integrity of the enzyme but not for the catalytic process itself. The maximum percentage of inhibition by thiol blocking agents is similar to the inhibition of HDCP hydrolyzing activity detected in CSA by those agents (*17*).

We previously reported that CSA is able to hydrolyze HDCP and *p*-NPB by a similar mechanism on the basis of the phosphorylation−dephosphorylation or acetylation−deacetylation of a tyrosine residue in the active center (*14*). The dephosphorylation process was found to be extremely slow (*14*). Carbarylase activity of RSA was inhibited by *p*-NPB in a competitive way (Table 2), demonstrating that *p*-NPB and carbaryl are hydrolyzed in the same active center and suggesting that this site might be the same as that where HDCP is hydrolyzed. RSA carbarylase activity was also inhibited by HDCP (Figure 2B). The time-dependent reactivation of carbarylase activity inhibited by HDCP (Figure 4) suggests that carbaryl and HDCP are hydrolyzed in the same active center by a comparable mechanism. Indeed, immediately after the incubation of RSA with HDCP, the active centers of most of the RSA molecules are phosphorylated by HDCP, causing an important inhibition of carbarylase activity. However, the enzyme is dephosphorylated slowly with time, and the carbarylase activity is recovered (Figure 4).

The amino acid sequence Arg-Tyr-Thr-Arg was identified as responsible for the specific labeling of BSA by the organophosphorus insecticide diisopropyl fluorophosphate (*15*). When the albumin amino acid sequence was known, the residue Tyr410 for BSA or Tyr411 for HSA was recognized as the residue acetylated during the hydrolysis of *p*-nitrophenyl acetate by these proteins. RSA displays the amino acid sequence Arg-Tyr-Thr-Lys (*14*). This sequence differs from BSA and HSA sequences only in the last arginine, which is substituted by lysine. Both arginine and lysine are basic amino acids and probably could play similar roles in a hypothetical hydrolysis of esters. Therefore, the sequence Arg-Tyr-Thr-Lys might be the target of the acetylation during *p*-NPB hydrolysis and the target of carbamylation during carbaryl hydrolysis.

All discussed data are consistent with a mechanism of hydrolysis on the basis of the transient carbamylation of the Tyr411 residue of RSA. This mechanism will be similar to the mechanism proposed for the hydrolysis of carboxyl esters (*10*) and HDCP (*14*) by serum albumins. The carbamylation of tyrosine will originate the release

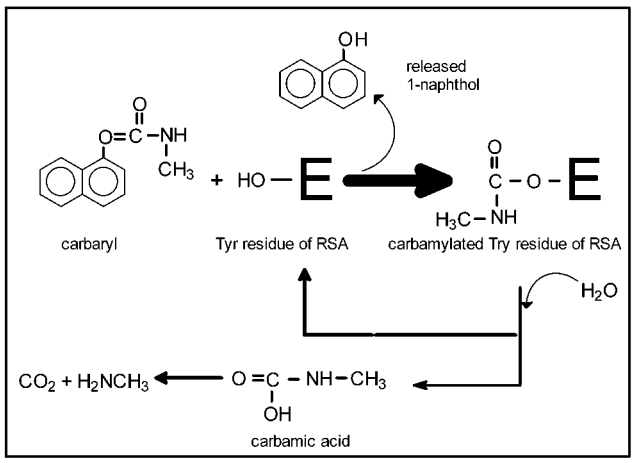

Figure 5. Proposed hydrolysis mechanism for the hydrolysis of carbaryl by RSA. In the first step, carbaryl reacts with a tyrosine residue of RSA, yielding free 1-naphthol and carbamylated RSA. In the second step, a water molecule attacks the carbamylated complex, releasing carbamic acid and free enzyme. The free enzyme is ready for a new catalytic cycle while carbamic acid quickly decomposes into CO_2 and methylamine.

of 1-naphthol, yielding a carbamylated RSA. The carbamoyl moiety will be further released by the attack of a water molecule. The released carbamic acid instantaneously decomposes into CO_2 and methylamine. The proposed mechanism is displayed in Figure 5. Nevertheless, our data cannot exclude the possibility that another active center in albumin can hydrolyze carbaryl at lower rate. This mechanism is similar to the mechanism described for serine esterases (like acetylcholinesterase) but with tyrosine instead of serine playing the most important role.

A very low K_{cat} (7.1×10^{-5} s^{-1}) was observed for the hydrolysis of carbaryl by RSA (Table 1). This low turnover means that, during 5 h of reaction (standard reaction time), each albumin molecule only interacts with around one molecule of carbaryl. It might suggest that the proposed carbamylation of the tyrosine residue might be nonreversible. However, this hypothesis is rejected because experiments (not shown) demonstrate that by prolonging the incubation time by more than 5 h, the amount of hydrolyzed carbaryl is increased and does not remain constant, as can be anticiped if a nonreversible carbamylation took place.

As was demonstrated previously the carbarylase activity of RSA displays a very low turnover number and is inhibited by fatty acids. However, the efficacy of albumins as detoxification system is based on the extraordinary large number of enzyme molecules. Indeed, serum albumin represents more than 50% of total protein in serum (*16*), and because the inhibition by fatty acids is not complete, this suggests the presence of a number of molecules with capability to hydrolyze carbaryl. In addition, the contribution of albumins to carbamate detoxification must not be considered only in serum because it represents only around 30% of the total albumin in the whole animal body (*18*). These extravascular molecules of albumin present different grades of binding to fatty acid depending on the tissue (gut, liver, muscle, or skin) and, therefore, might be present different grades of activity. Finally, the total amount of albumin present in the whole body is solely synthesized in the liver (*18*), and this protein is synthesized fatty acid-free; the liver is an important reservoir of detoxificating active enzyme and probably is the most important organ involved in the hydrolysis of carbamates via hydrolysis by albumins.

The promotion phenomenon is subject of maximum concern because we might be on the verge of elucidating the process of neurodegenerative diseases of as yet unknown ethiology. The necessity to develop studies about enzymes involved in the detoxification of promoters (carbamates) seems clear because the action of these enzymes might significantly reduce the amount of the promoter before it reaches the target of promotion in nervous systems.

Finally, it is remarkable that the capability of albumins to hydrolyze carbamates has strong ecotoxicological implications. Indeed, because albumin is universally present in all vertebrate species, it will be possible to design carbamate structures more susceptible to hydrolysis by albumins. In this way, the resistance of nontarget species (vertebrates) to the carbamate insecticides will be increased. This increase of resistance might be specially important in species lacking other disposition and metabolic systems.

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