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A COMPARISON OF THE DECARBAMOYLATION RATES OF PHYSOSTIGMINE-INHIBITED PLASMA AND RED CELL CHOLINESTERASES OF MAN WITH OTHER SPECIES

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Abstract—Plasma and red cells from a variety of animal species were used to demonstrate that there is a relationship between the decarbamoylation rates of physostigmine-inhibited plasma and red cell cholinesterases *in vitro* and the effectiveness of carbamate pretreatment against nerve agent poisoning reported in the literature. Decarbamoylation rates were faster in the non-human primates than in the guinea-pig, and carbamate pretreatment is more effective in these species than in the guinea-pig. The data for the decarbamoylation rates of physostigmine-inhibited enzymes suggests that the non-human primates are the best animal model for extrapolation of protection studies from animal species to man. Control values for red cell acetylcholinesterase (AChE) activity (μmol/min/mL blood) using acetylthiocholine (1 mM) were higher in the human (4.98) and the rhesus monkey (4.14) than in the marmoset (0.84) and the guinea-pig (0.83). Plasma cholinesterase (ChE) activity (μmol/min/mL plasma) using butyrylthiocholine (10 mM) was highest in the rhesus monkey (9.29), intermediate in human (5.10) and guinea-pig (6.06), and lowest in the marmoset (4.07). There was a species difference in the relative activity of AChE: ChE in blood, human (65:35), rhesus monkey (45:55), marmoset (30:70) and guinea-pig (20:80). The rate of recovery of red cell AChE and plasma ChE activities, following incubation of whole blood with physostigmine (1×10^{-7} M), was in the order human > rhesus monkey > marmoset > guinea-pig. During the incubation of red cells with physostigmine there was little recovery of AChE activity for 3–4 hr in any species. During the incubation of plasma with physostigmine there was complete recovery of ChE activity by 2–3 hr in the human and rhesus monkey and a significant recovery by 3 hr in the marmoset and guinea-pig. This suggests that a component of plasma, possibly ChE, was responsible for the degradation of physostigmine, presumably by hydrolysis. There was a marked species difference in the decarbamoylation rates of physostigmine-inhibited enzyme. In the red cell the $t_{1/2}$ values (min) were 14.8 (human), 21.2 (rhesus monkey), 17.9 (marmoset) and 31.9 (guinea-pig). In the plasma the $t_{1/2}$ values (min) were 11.2 (human), 32.9 (rhesus monkey), 44.1 (marmoset) and 52.4 (guinea-pig).

Decarbamoylation studies have been mainly restricted to enzyme sources such as cow erythrocytes, electric eel and fly head [1], and have not included mammalian species that are important when extrapolating carbamate pretreatment data from rodents and non-human primates to man. Protection studies have shown that pretreatment with a carbamate supported by therapy with an anticholinergic drug provides an effective treatment for poisoning by soman (1,2,2-trimethylpropyl methylphosphonofluoridate) in mouse, rats, guinea-pigs, rabbits [2–4] and primates [5]. The protective action of carbamates against organophosphorous poisoning depends on their ability to carbamoylate reversibly acetylcholinesterase (AChE: EC 3.1.1.7) [6–8]. The carbamoylated AChE would be protected from phosphonylation during subsequent poisoning with an organophosphate. Spontaneous decarbamoylation of the enzyme together with metabolism of excess organophosphate would release sufficient AChE to sustain life [2]. The rate of spontaneous decarbamoylation is dependent on the acyl group and the enzyme source [1].

There is a marked species difference in the protection afforded by carbamate pretreatment. Pyridostigmine pretreatment and atropine therapy protects rhesus monkeys > marmosets > non-primate species [5], whereas physostigmine pretreatment and atropine therapy protects guinea-pig > rabbit > mouse > rat [2]. Also, physostigmine and hyoscine pretreatment has been shown to be effective in the cynomolgus non-human primate [9]. The species differences observed in the effectiveness of carbamate pretreatment against organophosphorous poisoning reported in the literature may either be related to the rates of decarbamoylation of AChE and cholinesterase (ChE: EC 3.1.1.8) in the different species or may be due to different rates of metabolism and distribution of the carbamate in the body.

The aims of this study were: firstly, to monitor the recovery of AChE and ChE following incubation of whole blood, red cells and plasma of several species with physostigmine; secondly, to compare the rates of decarbamoylation of physostigmine-inhibited plasma and red cells in the various animal species to determine if a relationship exists between these rates of decarbamoylation and the species differences reported in the effectiveness of carbamate pretreatment against soman poisoning; and finally, to identify which species was most similar to man in this context.

MATERIALS AND METHODS

Acetylthiocholine, butyrylthiocholine and 5,5-dithiobis(2-nitrobenzoic acid) (Ellman reagent) were purchased from BDH Chemicals Ltd (Poole).

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Blood samples were taken by venepuncture from human volunteers (male), who gave their informed consent, rhesus monkeys (male and female) and marmosets (male and female pooled samples), and by heart puncture from guinea-pigs (male), and were dispensed into EDTA tubes. When appropriate, whole blood samples were centrifuged to separate plasma from cells. The cells were washed three times and made up to their original whole blood volume with 0.9% saline for use.

Human cholinesterase (serum-cholinesterase, Behringwerks AG, Marburg; Hoechst Pharma AG, Zurich) purified 10,000-fold [10] and equivalent to 500 mL plasma was diluted 1 in 500 with phosphate buffer (0.1 M, pH 7.4).

Assay for acetylcholinesterase and cholinesterase activities. Red cell AChE and plasma ChE activities were measured using a modified method of Ellman *et al.* [11]. Cells were diluted 1 in 500 (v/v) with phosphate buffer (0.1 M, pH 7.4) (human and rhesus monkey) and 1 in 200 (v/v) (marmoset and guinea-pig). Plasma and purified ChE were diluted 1 in 500 with 0.1 M pH 7.4 phosphate buffer. Samples were assayed at 30° using acetylthiocholine (1 mM) for red cell AChE and butyrylthiocholine (10 mM) for plasma ChE. (These substrate concentrations were optimal for the species studied based on previous work with a range of substrate concentrations.) The change in absorbance was measured every 30 sec for 3 min and was linear throughout this time. Enzyme activities were expressed as μmoles acetylthiocholine hydrolysed/min/mL whole blood or μmoles butyrylthiocholine hydrolysed/min/mL plasma. Enzyme activities following incubation with physostigmine were expressed as percentage inhibition of control.

Incubation of whole blood, washed red cells or plasma with physostigmine. Samples of whole blood, washed red cells, plasma or purified ChE were incubated with physostigmine (1×10^{-7} M) in a waterbath at 37°. This concentration of physostigmine was chosen because it produced a significant initial level of inhibition and the recovery of enzyme activity could be readily measured with time. Aliquots were removed at various time intervals. The whole blood was separated into plasma and cells for the assay of ChE and AChE activities. The red cell and the plasma and purified ChE incubates were used for the assay of AChE and ChE, respectively. Results were plotted as percentage inhibition against time in minutes.

Decarbamoylation study. Washed red cells, plasma or purified ChE were incubated with physostigmine (1×10^{-7} M) at 37° for 10 min. Cells were washed three times with 0.9% saline and diluted 1 in 500 (v/v) with phosphate buffer (0.1 M, pH 7.4) (rhesus monkey and human) and 1 in 200 (v/v) (marmoset and guinea-pig). Plasma and purified ChE was diluted 1 in 500 (v/v) with phosphate buffer (0.1 M, pH 7.4). All samples were placed in a waterbath at 37° and aliquots removed 3 min later and then at 6 min intervals for the measurement of activity of red cell AChE and at 10 min intervals for the measurement of plasma ChE activity. The percentage inhibition remaining was plotted on semi-logarithm paper against time (min) and the best line through

Table 1. Control values for red cell AChE and plasma ChE activities in different species

Species	N	RBC AChE (μmol/min/mL blood)*	Plasma ChE (μmol/min/mL plasma)†	Packed cell volume (%)	Plasma ChE (μmol/min/mL blood)†	AChE:ChE in blood
Human	6	4.98 ± 0.36	5.10 ± 0.24	49.0 ± 0.8	2.61 ± 0.12	65:35
Rhesus monkey	7	4.14 ± 0.30	9.29 ± 0.96	47.5 ± 3.0	4.93 ± 0.51	45:55
Marmoset	6	0.84 ± 0.04	4.07 ± 0.33	47.6 ± 0.9	2.16 ± 0.19	30:70
Guinea-pig	11	0.83 ± 0.05	6.06 ± 0.34	28.0 ± 4.6	3.76 ± 0.21	20:80
Purified human ChE	2	—	10.50	—	—	—

The results are expressed as the mean ± SEM.
* Acetylthiocholine (1 mM) as substrate.
† Butyrylthiocholine (10 mM) as substrate.

the points was drawn. The slope of the line was used to calculate K_{obs}. The $t_{1/2}$ value was calculated from K_{obs}. The values for $t_{1/2}$ were tested for significant differences using the Student's t-test, $P < 0.05$.

RESULTS

Control values for red cell AChE and plasma ChE activities

The control values for red cell AChE, plasma ChE and purified ChE activities are shown in Table 1. The values for red cell AChE activity were higher in the human and rhesus monkey than in the marmoset and guinea-pig. The values for plasma ChE activity were highest in the rhesus monkey with lower values in guinea-pig, human and marmoset. The value for purified ChE was similar to that for rhesus monkey plasma. The ratios of red cell AChE activity: plasma ChE activity in whole blood showed species differences (Table 1).

Incubation of whole blood with physostigmine

Incubation of whole blood with physostigmine (1×10^{-7} M) produced a maximum inhibition of red cell AChE of about 70%. Plasma ChE was similarly inhibited in all species except the guinea-pig (85%) (Fig. 1a and b). The red cell and plasma activities had returned to normal values within 3 hr in the human and within 5.5 hr in the rhesus monkey. The marmoset and the guinea-pig red cell AChE activities were still inhibited by 40% and the plasma ChE by 30% and 60%, respectively, at 5.5 hr.

Incubation of red cells or plasma with physostigmine

Incubation of red cells with physostigmine (1×10^{-7} M) produced a red cell AChE inhibition of 70–80% in all species. The AChE activity did not return to normal values in any species by 5.5 hr. The rate of recovery was faster in the human than in the rhesus monkey. There was little or no recovery in the marmoset or in the guinea-pig (Fig. 2a). Incubation of plasma with physostigmine (1×10^{-7} M) produced a plasma ChE inhibition of 50–60% in all species except the guinea-pig plasma which was inhibited by 78%. The human and rhesus monkey plasma ChE activity had returned to within normal values by 2.5 to 4 hr. The guinea-pig and marmoset plasma ChE activities were still inhibited by 15 and 30%, respectively, at 5.5 hr (Fig. 2b). Incubation of purified human ChE with physostigmine (1×10^{-7} M) produced an inhibition of 56%, ChE activity had returned to normal by 2 hr.

Decarbamoylation rates for physostigmine-inhibited red cells and plasma

Physostigmine inhibited red cell AChE to a similar extent in all species, (63–74%) (Table 2). Plasma ChE was slightly less inhibited (52–57%) but showed a greater variation in degree of inhibition within species (Table 2). Decarbamoylation followed first order kinetics in all species (Fig. 3a and b). The $t_{1/2}$ values (min) for red cell AChE were 14.8 (human), 17.9 (marmoset), 21.2 (rhesus monkey) and 31.9 (guinea-pig). The values were all significantly different ($P < 0.05$) between species. The $t_{1/2}$ values

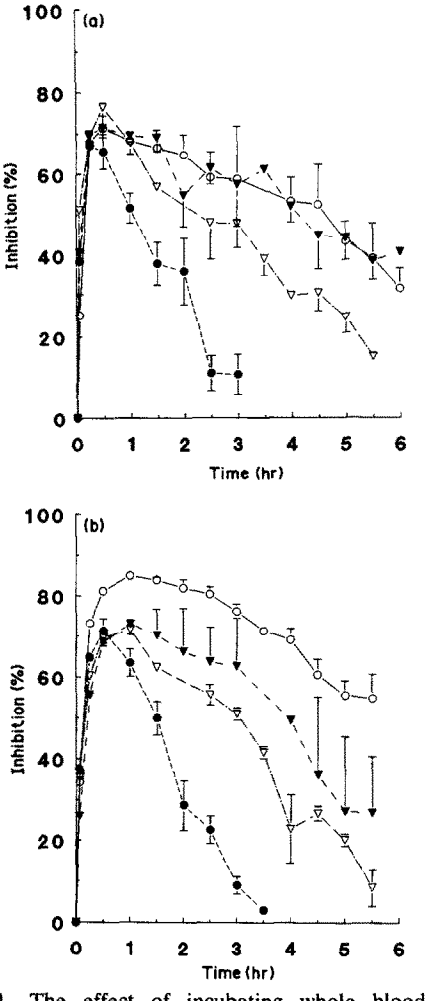

Fig. 1. The effect of incubating whole blood with physostigmine (1×10^{-7} M) and measuring the inhibition of (a) red cell acetylcholinesterase, (b) plasma cholinesterase in guinea-pig (—○—), human (—●—), rhesus monkey (—▽—) and marmoset (—▼—). Each point represents the mean ± SEM of 2–5 experiments.

(min) for plasma ChE were 11.2 (human), 32.9 (rhesus monkey), 44.1 (marmoset) and 52.4 (guinea-pig). Values for the human were significantly different ($P < 0.05$) from all other species and values for the rhesus monkey were significantly different ($P < 0.05$) from the guinea-pig. Purified human ChE was inhibited by 69% and the $t_{1/2}$ value was 35.6 min (Table 2).

Comparison between decarbamoylation rates and the effectiveness of carbamate pretreatment against soman poisoning

The decarbamoylation rates for physostigmine inhibited enzymes were faster in the non-human primates than in the guinea-pig. Pyridostigmine pretreatment is reported to be more effective in the non-human primates than in the guinea-pig. Physostigmine and hyoscine pretreatment is reported to be more effective than pyridostigmine pretreatment both in the guinea-pig and the cynomolgus

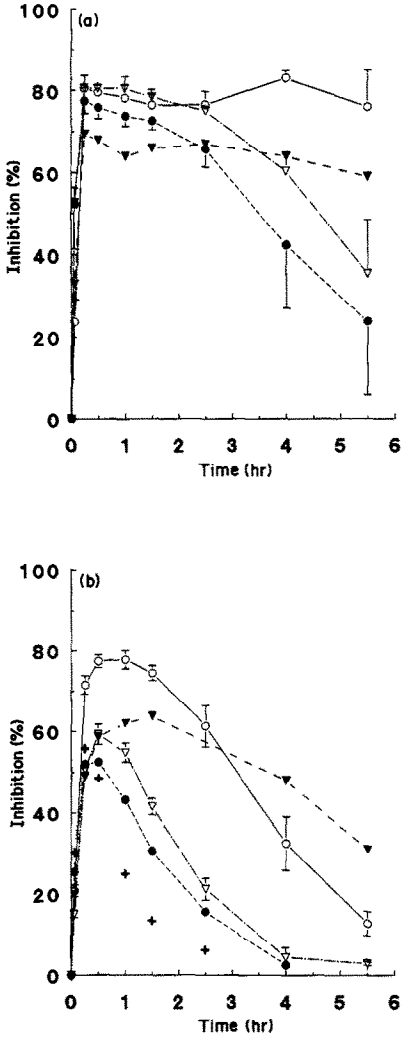

Fig. 2. The effect of incubating red cells or plasma with physostigmine (1×10^{-7} M) and measuring the inhibition of (a) red cell acetylcholinesterase and (b) plasma cholinesterase in guinea-pig (—○—), human (—●—), purified human cholinesterase (—+—), rhesus monkey (—▽—) and marmoset (—▼—). Each point represents the mean of 2–3 experiments ± SEM.

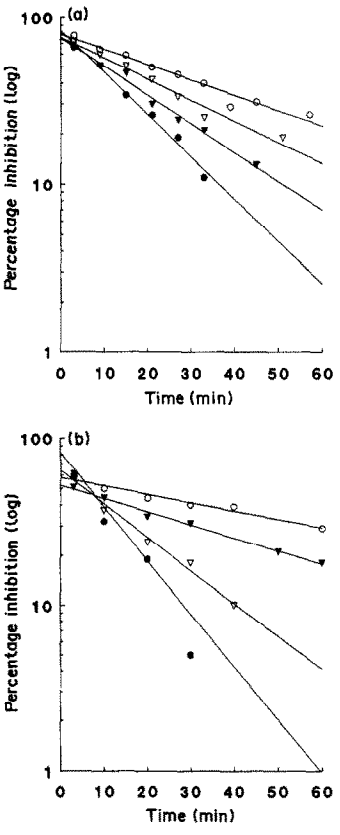

Fig. 3. Graph to show the typical rate of decarbamoylation of physostigmine- (1×10^{-7} M) inhibited (a) red cell acetylcholinesterase and (b) plasma cholinesterase in guinea-pig (—○—), human (—●—), rhesus monkey (—▽—) and marmoset (—▼—).

monkey. It is not possible to state whether the physostigmine pretreatment is more effective in the non-human primate than in the guinea-pig because comparative studies have not been reported in the literature.

DISCUSSION

This study has clearly shown that there is a marked

Table 2. Decarbamoylation rates for physostigmine-inhibited red cells and plasma of different species

	Red cells				Plasma			
Species	N	% Inhibition (at 3 min)	k_{obs}/sec × 10^{-4}	$t_{1/2}$ (min)	N	% Inhibition (at 3 min)	k_{obs}/sec × 10^{-4}	$t_{1/2}$ (min)
Human	11	65.6 ± 0.9	8.1 ± 0.6 (11.5–6.4)	14.8 ± 0.9 (10.0–17.9)	6	52.8 ± 7.6	15.9 ± 3.9 (26.8–5.3)	11.2 ± 3.1 (4.3–21.6)
Rhesus monkey	9	70.4 ± 0.8	5.5 ± 0.3 (7.1–4.9)	21.2 ± 0.9 (16.3–23.7)	7	52.0 ± 3.9	5.6 ± 1.5 (11.5–1.9)	32.9 ± 7.7 (10.0–60.5)
Marmoset	7	63.1 ± 2.5	6.5 ± 0.4 (7.6–5.3)	17.9 ± 0.9 (15.1–22)	6	52.0 ± 2.4	3.1 ± 0.6 (5.8–1.6)	44.1 ± 7.6 (19.9–70.6)
Guinea-pig	11	74.2 ± 1.5	3.7 ± 0.1 (4.4–2.6)	31.9 ± 1.4 (26.3–43.7)	11	57.1 ± 2.2	2.4 ± 0.2 (3.9–1.6)	52.4 ± 4.5 (29.6–71.6)
Purified human ChE		—	—	—	2	69	3.3	35.6

The results are expressed as the mean ± SEM with the range in brackets.

species difference in the red cell AChE and plasma ChE activities for the human, rhesus monkey, marmoset and guinea-pig. There is also a species difference in the ratio of red cell AChE activity: plasma ChE activity in whole blood with the red cell AChE contribution being greater in the human than in the guinea-pig in particular.

Incubation of whole blood with physostigmine produced a marked species difference in the time taken for red cell AChE and plasma ChE activities to return to normal values. The rate of recovery of both enzymes was faster in human blood than in rhesus monkey and marmoset blood which were faster than in guinea-pig blood. Also, the rates of spontaneous decarbamoylation of physostigmine-inhibited red cells and plasma were markedly different in the species studied. The $t_{1/2}$ value was shorter in the human than in the rhesus monkey and marmoset, which were shorter than that in the guinea-pig.

It is likely that a component of the plasma, possibly ChE, degrades physostigmine to some extent in all the species studied. This was evident because when plasma was incubated with physostigmine, the plasma ChE showed a significant recovery of activity with time, whereas, when red cells were incubated with physostigmine, AChE showed little or no recovery for 3 to 4 hr. However, when whole blood was incubated with physostigmine, the red cell AChE activity also showed a significant recovery in a shorter time. Furthermore, when a sample of purified human serum cholinesterase was incubated with physostigmine the ChE activity recovered at a similar rate to human plasma. Other studies have suggested [12–14] that the component of plasma responsible for physostigmine breakdown is ChE itself—carbamoylation of the active site followed by decarbamoylation, would result in the hydrolysis of the carbamate.

The time taken for the recovery of red cell AChE and plasma ChE activity following incubation of whole blood with physostigmine was in the order human faster than rhesus monkey, which was faster than marmoset, which was faster than guinea-pig. Other workers [15] have reported similar inhibition levels and recovery times for human whole blood with the same concentration of physostigmine. The species differences observed in this study may be due either to differences in the concentration of ChE present in the plasma or to the ratio of AChE : ChE. However, the control values for plasma ChE activity were the same in the human and in the guinea-pig and the contribution of ChE in whole blood is lower in the human (35%) than in the guinea-pig (80%). This would suggest that plasma ChE is not solely responsible for the disappearance of physostigmine from the blood, unless physostigmine, unlike butyrylthiocholine, is hydrolysed at a faster rate by human plasma ChE than by guinea-pig plasma ChE. Also, the difference in the rates of recovery does not correlate with the initial level of inhibition produced by physostigmine. The guinea-pig plasma ChE showed a higher maximum inhibition than the human, but marmoset plasma ChE was inhibited to the same extent as the human and still took longer to return to control values.

The rates for the spontaneous decarbamoylation of physostigmine-inhibited red cells and plasma also showed marked species differences: the rates were fastest in the human and slowest in the guinea-pig, with the rhesus monkey and marmoset producing intermediate rates. This difference in decarbamoylation rates may explain the species difference in enzyme recovery rates of whole blood incubated with physostigmine, although when plasma was incubated with physostigmine human and rhesus monkey ChE recovered at a similar rate. The plasma $t_{1/2}$ values were significantly different in the human and rhesus monkey, but the value for purified human ChE was the same as in the rhesus monkey. The values for rhesus monkey red cells were similar to those previously quoted for bovine erythrocytes under similar conditions [16–18], but comparison with other data was difficult because of different temperatures and pH values used for the incubation and the assay.

There was a large variation in the decarbamoylation rates for physostigmine-inhibited plasma within each species, whereas in the red cell the rates varied less. This difference between the consistency of results in the red cells and plasma may be due to the fact that excess physostigmine would have been removed by washing the red cells, prior to dilution, whereas plasma could not be washed and was diluted in the usual way. This dilution should be sufficient to decrease the inhibitor concentration and prevent carbamoylation [6, 17, 19, 20]. The roles of plasma ChE and red cell AChE in the body are still not fully understood, but the red cell AChE is considered to be a better indicator for the functional AChE, which has an important role at the nerve endings. The red cell decarbamoylation rates were more consistent within the species studied.

There is a species difference in the protection afforded by carbamate pretreatment against soman poisoning. Pyridostigmine pretreatment and atropine therapy protects rhesus monkeys > marmosets > non-primate species [5], while physostigmine pretreatment and atropine therapy protects guinea-pigs > rabbit > mouse > rat [2]. Physostigmine and hyoscine pretreatment affords complete protection against soman poisoning in the cynomolgus non-human primate [9]. The present study has demonstrated a species difference in the rates of spontaneous decarbamoylation of physostigmine-inhibited red cell AChE and plasma ChE. The red cell decarbamoylation rates were faster in the non-human primates than in the guinea-pig, and pyridostigmine pretreatment is more effective in the non-human primates than in the guinea-pig. The rate of decarbamoylation for human red cells inhibited with physostigmine was similar to that obtained in rhesus monkey and in marmoset, which suggests that carbamate pretreatment might also be effective in the human.

The relative effectiveness of carbamate pretreatment and therapy with an anti-cholinergic for nerve agent poisoning is dependent on number of factors. First, the level of carbamoylated AChE and the rate of decarbamoylation. Secondly, the metabolism and the rate of disappearance of the free nerve agent.

Thirdly, the binding of nerve agent to various sites including plasma proteins like carboxylesterase and finally, the metabolism and duration of action of the therapeutic drugs.

Other workers [21, 22] have demonstrated a species difference in the plasma concentration of a soman scavenger, carboxylesterase, which may explain the species differences in the effectiveness of carbamate pretreatment. They demonstrated that if carboxylesterase was inhibited then the protection afforded by carbamate pretreatment was similar in the rat, guinea-pig and rabbit. Human plasma is reported to contain very little carboxylesterase activity [23] which is probably similar to the zero and minor levels reported for the rhesus monkey and the marmoset [21]. The human could therefore be expected to respond to carbamate pretreatment in a similar way to the non-human primates.

In conclusion, this study has clearly shown that there is a species difference in the rates of decarbamoylation of physostigmine-inhibited AChE and ChE. The differences in these rates, together with other factors, may explain the species differences reported in the literature for the effectiveness of carbamate pretreatment against nerve agent poisoning. Also, the human has similar decarbamoylation rates for physostigmine-inhibited red cell AChE to the non-human primates and on this basis the non-human primate is the best model for extrapolating protection studies from animal species to man.

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