Arch. Environ. Contam. Toxicol. 37, 283–289 (1999)

ARCHIVES OF
Environmental
Contamination
and Toxicology

Oxime Reactivation of RBC Acetylcholinesterases for Biomonitoring

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Received: 8 July 1998/Accepted: 3 May 1999

Abstract. A low-variability method to reactivate blood cholinesterases (ChEs) after prior exposure of mammals, including humans, to ChE-inhibiting organophosphate esters (OPs) is presented. A concentration of 10 mM pyridine 2-aldoxime methochloride (2-PAM Cl) was incubated with intact red blood cells (RBCs) and assayed virtually free of interfering oxime and hemoglobin (Hb). Variability was decreased by reducing the number of washing steps and sedimenting RBC ghosts through a 7% sucrose cushion. Statistically significant detections of reactivations as low as 5% with average ''false positives'' of 3.8% were achieved. Relative rates and extent of reactivation after OP treatment of rabbit RBC AChE *in vitro* were of the order dimethyl- (DDVP) > diethyl- (ethyl paraoxon) >, di*iso*propyl-substituted (di*iso*propyl fluorophosphate; DFP) OPs. Rabbit RBC AChE was reactivatable for up to 60 h following dermal exposure to ethyl parathion and reactivatable for only 12 to 24 h following exposure to methyl parathion. Reactivation of plasma ChEs with 0.1 mM 2-PAM Cl in the same animals was achievable for only 12 to 24 h after ethyl parathion and for only 1 to 4 h after methyl parathion.

Oximes such as pyridine 2-aldoxime methochloride (2-PAM Cl) reactivate OP inhibitions of ChEs providing ''aging'' (loss of an alkyl group from the OP-enzyme complex) has not occurred (Taylor 1996). Research on oximes has been directed toward their use as antidotes, especially for OP chemical warfare agents (reviewed by Moore *et al.* 1995) and biochemical mechanisms of aging and reactivation (Wilson *et al.* 1992) even in cell cultures (Funk *et al.* 1994). Only a few studies have utilized oximes to assess OP exposures to wildlife (invertebrates, birds, mammals), livestock, or humans (*e.g.*, Karlog and Poulsen 1963; Martin *et al.* 1981; Hooper *et al.* 1989; Lifshitz *et al.* 1994; McCurdy *et al.* 1994; Sanchez-Fortun *et al.* 1996). This paper describes a simple method to quantitatively reactivate OP-inhibited mammalian RBCs AChE.

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Materials and Methods

Chemicals

Chemicals and reagents were prepared with double-glass-distilled, deionized water. Buffers for whole blood or washed RBCs were 300 mOsm (Advanced, Wide-Range Osmometer 3W2; Advanced Instruments, Inc., Needham Heights, MA). Unless otherwise noted, positive displacement Eppendorf Repeater pipettes (Brinkmann Instrument, Inc., Westbury, NY) and 0.5 ml Combitips were used for pipetting blood and other reagents. Sources of chemicals were: acetylthiocholine iodide (AThCh), di*iso*propylfluorophosphate (DFP), and 5,5′-dithio*bis*-[2-nitrobenzoic acid] (DTNB; Sigma Chemical Co., St. Louis, MO); 2-PAM Cl and paraoxon (*O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphate; Aldrich Chemical Co., Milwaukee, WI); Dichlorvos (*O*,*O*-dimethyl-*O*-2, 2-dichlorovinyl phosphate; (DDVP), ethyl parathion (*O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate), and methyl parathion (*O*,*O*-dimethyl-*O*-*p*-nitrophenyl phosphorothioate; Chemical Services Co., West Chester, PA). Paraoxon was redistilled by M. McChesney (Environmental Toxicology Department, University of California, Davis). Sources of reagents were: sodium phosphate buffers and sodium chloride (Aldrich), Triton-X 100 (scintillation grade, Amersham Corp, Arlington Heights, IL), potassium chloride (Merck Chemical Co., Rahway, NJ), sucrose (Mallinckrodt Inc., St. Louis, MO), and sodium heparin (Invenex, LyphoMed, Inc., Rosemont, IL; 1,000 USP units/ml). Bovine serum albumen (BSA; Gibco Laboratories, Grand Island, NY; crystalline electrophoresis grade: 99% purity) was free of measurable ChE activity.

Animals

Adult female New Zealand white rabbits (*Oryctolagus cuniculus*), a common animal for dermal toxicity studies, large enough for sequential blood samplings, were 1 to 2 years old and weighed 4.8 to 6.6 kg. Animal care and research protocols were reviewed and approved by the campus veterinarian.

Blood Handling

Blood was drawn with heparin-treated syringes through 25-gauge needles and transferred to heparin-treated 5-ml disposable plastic tubes. Duplicate hematocrits were determined for each sample using an IEC HB-5 centrifuge fitted with an IEC 927 microhematocrit head (International Equipment Company; Needham Heights, MA). Plasma was separated from whole blood by centrifugation (Sorvall RC-2B

Automatic Refrigerated Centrifuge using the outer wells of a Sorvall SM-24 head) at 1,100 *g* for 10 min at 4°C and was removed by aspiration. In cases where washed RBCs were needed prior to reactivation, for example, after *in vitro* OP treatments, RBCs were washed by adding 5 ml of RBC Wash Buffer (135 mM NaCl, 20 mM sodium phosphate buffer, 3 mM KCl, pH 8.0) to each sample tube, resuspending the pellets, and centrifuging again at 1,100 *g* for 10 min at 4°C. The supernatant was discarded, the procedure repeated three times, and the volume of the washed RBCs brought up to the original with RBC Wash Buffer. Hematocrits were used to correct AChE activities for the relatively higher hematocrits of whole blood (RBCs pack less tightly (Albert *et al.* 1965) due to the relatively viscous plasma).

RBC AChE Oxime Reactivation Assay

Figure 1 is a flowchart of the reactivation method modified from Wilson *et al.* (1992). Two 50-µl aliquots of whole blood (or washed RBCs if RBCs were treated with OPs *in vitro*) were pipetted into weighed, 10-ml round-bottom polycarbonate, high-speed centrifuge (HSC) tubes containing 900 µl of Reactivation Buffer (195 mM monobasic and 130 mM dibasic sodium phosphate buffer, 3 mM KCl, pH 8.0). Duplicate hematocrits were taken, and each tube was checked to ensure it was free of clumped or clotted blood. Reactivations were initiated by pipetting 50 µl of 2-PAM Cl (200 mM in 160 mM NaCl; 10 mM final concentration) into one tube, pipetting the same volume of 160 mM NaCl into another tube, and incubating the tubes in a shaking waterbath (40–60 cycles/min) at 25°C for 40 to 60 min.

The method of Dodge *et al.* (1963) was modified to prepare RBC ghosts. Following oxime incubation, 5 ml of RBC Wash Buffer was added to each tube, and the RBCs were sedimented by centrifugation at 2,500 *g* for 15 min at 4°C. The clear, colorless supernatant was removed, each tube was vortexed vigorously to break up the tightly packed RBCs, and 5 ml of chilled Hemolyzing Buffer (20 imOsm sodium phosphate buffer, pH 7.4) was added. The contents were vortexed again, approximately 1.5 ml of a sucrose cushion (7% sucrose in Hemolyzing Buffer [w/v]) was added to the bottom of each tube with a 9-inch Pasteur pipet, and the tubes were centrifuged at 27,800 *g* for 20 min at 4°C. All but about 250 µl of the supernatant and sucrose cushion were removed without disturbing the pellet, assisted by a bright light behind the tube. Approximately 650 µl of Solubilization Buffer (chilled 0.5% Triton-X 100 in 0.1 M sodium phosphate buffer [v/v], pH 8.0) was added, and each tube was gently vortexed to dissolve the pellet away from the side of the tube. Enough additional Solubilization Buffer was added to each tube to yield 1.000 ± 0.005 g of contents. The tubes were gently vortexed and placed on ice for 15 min before measuring AChE activity. The entire method required approximately 2 h to complete, during which time samples were kept on ice for all but the 40- to 60-min reactivation reaction incubation, which was performed at 25°C.

Plasma ChE Reactivations

Plasma ChEs were reactivated to compare this method to the RBC AChE oxime reactivation assay by a modification of the method of Hooper *et al.* (1989) using 0.1 mM 2-PAM Cl to reduce its interference during AChE activity determinations. Reactivations were performed at 25°C rather than 38°C, and 0.3% BSA was added to the plasma dilution buffer to stabilize the ChE and reduce loss of enzyme activity. Plasma samples were either reactivated within 24 h of sampling or frozen at −70°C. There was no loss of ChE activity or reactivatability if the samples were processed within 2 months.

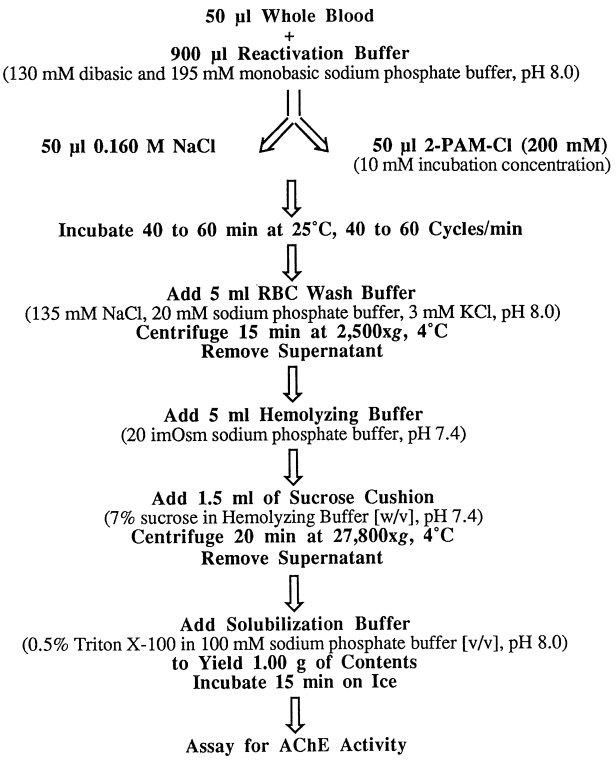

Fig. 1. Flow diagram of RBC AChE Oxime Reactivation Assay, modified from Wilson *et al.* (1992)

ChE Activity Measurements

The assay of Ellman *et al.* (1961) was performed in triplicate for each sample using a 96-well automated microplate reader (Bio-Tek EL340I) with temperature control. Assay volumes of 320 µl consisted of 250 µl of 0.1 M sodium phosphate buffer, pH 8.0, 10 µl of DTNB (5.15 mM in 0.1 M, pH 7.0 sodium phosphate buffer; 322 µM final concentration), 30 µl of enzyme sample, and 30 µl of AThCh (10.7 mM in 0.1 M, pH 8.0 sodium phosphate buffer; 1.0 mM final concentration). Substrate blanks consisted of 280 µl of 0.1 M sodium phosphate buffer, pH 8.0, 10 µl of DTNB, and 30 µl of AThCh. Assay plates were warmed to 25°C before each run. Temperature was monitored immediately before and after each run. Samples were added only to rows B through F of the plates because wells in rows A and H were as much as 1.5°C warmer than the rest. Unused wells were filled with 320 µl of blank constituents or buffer to further insulate sample-containing wells. ChE activities/ml were calculated using a standard extinction coefficient of 13,600 L mol^{-1} cm^{-1} and path length of 0.879 cm (Willard *et al.* 1981). RBC AChE activities/ml were divided by an average of duplicate hematocrits yielding moles AThCh hydrolyzed/min/ml packed RBCs. Plasma ChE activities were expressed as moles AThCh hydrolyzed/min/ml plasma.

Reactivation Assay Variability

The magnitude of reactivation sufficient to limit ''false positives'' to 5% or less was established by calculating a reactivation ratio $|(-1 + [\text{Oxime Treated ChE Activity}]/[\text{Untreated ChE Activity}]) \times 100|$% and subjecting it to a one-tailed student's *t* test for paired variates (Sokal and Rohlf 1969). Table 1 shows percent false positive

Table 1. Percent false positive frequency for untreated rabbit RBCs subjected to the RBC oxime reativation methodology[a]

Pipet	I A = 3 and B = 95%	II A = 3 and B = 99%	III A = 5 and B = 95%	IV A = 5 and B = 99%	V A = 5 and B = 99.5%
Eppendorf Repeater	13.5	7.7	3.8	1.9	0.0
Rainin EDP	13.5	3.8	1.9	0.0	0.0

[a] A = Percent reactivation ratio; B = confidence interval from one-tailed Student's *t* test for paired comparison, with two degrees of freedom, n = 52

values resulting from experiments employing various combinations of reactivation ratios and *t* test confidence intervals. In one experiment, the reactivation assay was performed 52 times with whole blood obtained over the course of 2 weeks from a single untreated female rabbit comparing the reliability of preparing the RBC ghosts with the positive pressure, Eppendorf Repeater, and the forced air, Rainin Electronic Digital Pipette (EDP) (Rainin Instrument Co., Inc., Woburn, MA). (An Eppendorf Repeater pipet was used to deliver reagents to assay wells.) In another experiment 42 untreated blood samples from three rabbits (the controls from the ''*in vivo* rabbit dosing experiments''), were collected over the course of 360 h at: 0, 1, 4, 8, 12, 24, 36, 48, 60, 72, 84, 108, 132, and 360 h.

Incubation Time versus Percent Reactivation

Experiments with DDVP-, paraoxon-, and DFP-inhibited rabbit RBC AChE were performed to determine the incubation time required for complete reactivation. Inhibitions with these OPs have distinct reactivation profiles characteristic of dimethyl-, diethyl-, and di*iso*propyl-phosphorylated AChEs, respectively (Clothier *et al.* 1981; Boskovic 1981; Langenberg *et al.* 1988). Three whole blood samples were withdrawn from three individual rabbits. Four 2-ml aliquots from each sample were pipetted into separate 10-ml HSC tubes. A 20-µl aliquot of inhibitor (final concentrations of 100 µM DDVP, 10 µM paraoxon, or 10 µM DFP in 160 mM NaCl) or 160 mM NaCl controls was added to each sample. Tubes were incubated for 30 min at 4°C and then washed four times as described in blood-handling procedure. Duplicate hematocrits were obtained for each sample, and the reactivation assay was performed. All samples were incubated with 10 mM 2-PAM Cl. Two uninhibited zero time tubes were prepared for each of three rabbit blood samples, receiving 20 µl saline rather than 2-PAM Cl. Reactivation reactions were incubated for 0 (no oxime added), 20, 40, 60, 180, and 360 min after adding 10 mM 2-PAM Cl (final concentration).

In Vivo *Rabbit Dosing Experiments*

Experiments were performed to determine the time-frame during which dimethyl- and diethyl-phosphorylated rabbit RBC AChE and plasma ChEs could be successfully reactivated following dermal exposure. It is known that dimethyl-phosphorylated ChEs, produced by methyl parathion, are readily reactivated, but age and spontaneously reactivate at high rates. Diethyl-phosphorylated ChEs, produced by ethyl parathion, are fairly easily reactivated and have slow rates of aging and moderate rates of spontaneous reactivation (Wilson *et al.* 1992). A 7.5-h dermal exposure period was employed to mimic exposures to agricultural workers.

Animal Preparation and Dosing

Three female rabbits were dermally dosed with each compound using a modification of the method of Knaak *et al.* (1980, 1984a, 1984b) to determine the time course of reactivation, to discover the effects of sequential blood withdrawal on rabbit RBC AChE activity, and to screen for ''false positives.'' Three rabbits served as controls. Each dosed rabbit received either 1 mg/kg of ethyl parathion or 5 mg/kg of methyl parathion in 95% ethanol. A rectangular patch of fur just below the shoulder and to the right of the spine was shaved from the back of each rabbit to represent 3% of the animal's total surface area (A: cm^2), with the length along the spine being twice the width using the following equation: $A = 9.5\ [\text{weight}^{0.66}]$ (weight in g; Harkness and Wagner 1989). Immediately before dosing, 1.5 ml of blood was withdrawn from each rabbit via an ear vein or artery. Duplicate hematocrits were obtained, and the sample was subjected to the RBC AChE Reactivation Assay. The animal of the least weight received 0.80 ml of the inhibitor solution, or ethanol for controls, while larger animals received proportionately more. Diluted inhibitor was applied and spread with a 1 ml plastic pipet tip and allowed to dry. Dosed rabbits were subsequently fitted with Elizabethan collars (412 Saf-T Shield, 12 inch, Ejay International, Inc, Glendora, CA) and placed into metabolism cages. Food and water were provided *ad libitum.* After 7.5 h, each OP was removed by washing with several pieces of ethanol-saturated cotton gauze, with 2.4 mg/kg (2.6 ± 0.3 mg/kg was not removed) of the 5 mg/kg methyl parathion dosage and 0.33 mg/kg (0.67 ± 0.05 mg/kg was not removed) of the 1 mg/kg ethyl parathion dosage being removed, as revealed by residue analyses (Weisskopf 1990).

RBC AChE Reactivations

Approximately 1.5 ml of blood was withdrawn before and 1, 4, 8, 12, 24, 36, 48, 60, 72, 84, 108, 132, and 360 h following dosing and assayed for reactivation. The reactivation assay required 200 µl of whole blood for each sampling period, including two 50-µl aliquots for 2-PAM Cl reactivation and AChE activity determinations and two 50-µl aliquots for hematocrits. For the remainder of the blood obtained, the plasma was separated as previously described; 150-µl aliquots of plasma were frozen at −70°C for later plasma ChE reactivation and activity determinations and 500 µl for gas chromatographic analyses (Weisskopf 1990).

Results

RBC Reactivation Assay Variability

The greater precision of the Eppendorf Repeater generated lower intrasample deviations than the Rainin EDP pipetter for 52 samples, yielding a higher level of false positives (Table 1). The greater precision of the Repeater versus the EDP was confirmed by weighing multiple 50-µl aliquots of distilled water (20°C) dispensed by each pipetter (data not shown). The Eppendorf Repeater pipette, a 5% reactivation ratio, and a 95% confidence interval were chosen for the remaining experiments. There was only one false positive (2.4%) at the 5% ratio, 95% confidence interval, from 42 samples withdrawn from three untreated control rabbits and sampled periodically over 360 h using the Repeater to aliquot solubilized AChE. Activities of 2-PAM Cl–treated samples were not significantly different from untreated samples. RBC AChE activities did not change significantly with sequential sampling.

Incubation Time versus Percent Reactivation

Table 2 shows the percent of dimethyl (DDVP), diethyl (paraoxon), and di*iso*propyl-phosphorylated (DFP) rabbit RBC AChE activity reactivated following incubations of various times with 10 mM 2-PAM Cl at 25°C. No aging (*i.e.*, loss of reactivatability) was detected with any of the phosphorylated enzymes. It was difficult to obtain complete AChE inhibition with DDVP, in part because a significant amount of spontaneous reactivation occurred during the RBC washings (data not shown). RBC AChE inhibited 51.3% with DDVP was reactivated almost completely during the first 20 min of incubation with 2-PAM Cl. Between 60 and 360 min were required to completely reactivate enzyme inhibited 95.4% with paraoxon. Approximately 63.7% reactivation of AChE occurred during the first 20 min of reactivation. An additional 26.5% reactivated over the next 40 min. Enzyme inhibited 38.5% with DFP required between 180 and 360 min to reactivate completely. We felt that a reactivation reaction incubation time greater than 1 h would be impractical, at least for environmental monitoring. For RBC AChE inhibited by an OP like DFP or a nerve warefare agent, an oxime such as obidoxime rather than 2-PAM Cl would result in a more rapid reactivation.

Table 2. Reactivation of phosphorylated AChE with 2-PAM Cl following *in vitro* treatment of rabbit RBCs[a]

	DDVP[b]	Paraoxon[b]	DFP[b,c]
Incubation Time (min)	Percent Initial Activity SE	Percent Initial Activity SE	Percent Initial Activity
0	48.7 ± 2.9	4.6 ± 2.8	61.5
20	97.2 ± 4.3	68.3 ± 1.3	67.8
40	97.1 ± 3.6	80.9 ± 1.2	79.7
60	95.7 ± 2.7	94.8 ± 2.1	79.8
180	104.5 ± 2.2	98.1 ± 0.9	87.8
360	98.8 ± 3.3	101.3 ± 1	102.5

[a] Percent initial activity values are means ± standard errors of the mean (SE) from samples obtained from three individual rabbits treated *in vitro*. All DDVP and paraoxon values were statistically significant compared to 0 min time points at ≥ 5% reactivation ratio and $p \leq 0.05$ from paired Student's *t* test

[b] Pesticide concentrations were: 100 µM DDVP, 10 µM paraoxon, and 10 µM DFP for 30 min at 4°C

[c] DFP data represent averages from blood of two individual rabbits treated *in vitro*

In Vivo *Rabbit Dosing Experiments*

Figure 2 shows reactivations for *in vivo* dermal OP applications. Only minor clinical signs were observed immediately after a 7.5-h dermal treatment with either 1 mg/kg ethyl parathion or 5 mg/kg methyl parathion. By visual observation it was determined that mild diarrhea occurred in all and lethargy in a few of the animals, and food and water consumption were much reduced for the first 12 to 24 h after treatment. Inhibition of RBC AChE increased over time (Figure 2A) even after removal of unabsorbed inhibitor. Maximal inhibition of RBC AChE (61.6 ± 8.3%) occurred within 12 h of dermal application of 1 mg/kg of ethyl parathion (Figure 2A). Recovery of AChE activity (presumably due to spontaneous reactivation) was first detected between 12 and 24 h following application (4.5 to 16.5 h after removal of the OP). The decrease in the recovery of the unreactivated activity curve after approximately 72 h may reflect the point where spontaneous reactivation ceased and appearance of newly formed RBCs became the primary source of AChE recovery. Chemical reactivation of RBC AChE was significant in all three rabbits sampled 1 to 60 h after dosing; reactivation of samples withdrawn 72, 84, 108, and 132 h following ethyl parathion administration were significant in one rabbit. Methyl parathion at 5 mg/kg was much less effective an inhibitor of RBC AChE than 1 mg/kg ethyl parathion. Maximum inhibition was only 12.7 ± 6.8% 8 h after dermal administration (Figure 2B). RBC AChE reactivations were significant in all three rabbits 4 h following application. Reactivations were significant at the 1, 8, and 12 h sampling periods for two of the three rabbits dosed. Apparent reactivations were obtained 24, 60, and 360 h after dosing in a single rabbit, but these may be false positives, especially at the 360 h time point. Rabbit plasma ChE was inhibited 68.2 ± 2.3% after 12 h of dermal exposure to 1 mg/kg of ethyl parathion (Figure 2C). A significant amount of activity returned over the subsequent 48 h, especially during the first 24 h. Due to the relatively rapid *de novo* synthesis of plasma ChE, 5- to 7-day half-life in humans (Whittaker 1986; Brock *et al.* 1990), it was difficult to determine the extent of spontaneous reactivation. All rabbits dosed with ethyl parathion showed significant plasma ChE reactivations 4 to 12 h after application. Two rabbits had significantly reactivatable plasma ChEs from 4 to 24 h following administration, whereas one yielded significant reactivations from 4 to 60 h after dosing. There was a maximal mean plasma ChE inhibition of 13.2 ± 7.8% 8 h following dermal application of 5 mg/kg methyl parathion (Figure 2D). Dimethyl-phosphorylated plasma ChEs were difficult to reactivate, as was the case for the RBCs. Significant reactivations were found for all three rabbits after 1 h, for two of three rabbits after 4 h, and for one rabbit 8 and 12 h after dosing. Some results were reviewed previously in preliminary form (Wilson *et al.* 1992).

Discussion

ChE reactivation assays are useful to assess the likelihood that recent significant exposure to an OP has occurred, especially when baseline blood ChE levels are lacking. Examples might be an animal brought to a wildlife care center or a patient brought to an emergency clinic displaying symptoms of cholinesterase poisoning. Advantages of using RBC AChE in reactivation assays include: (1) Mammalian RBCs can be treated with a high oxime concentration, optimizing rates of reactivation, followed by a wash step to remove the oxime, whereas a lower oxime concentration must be utilized with the plasma assay due to oxime induce ChE inhibition and AThCh hydrolysis. (2) RBC AChE is often more easily reactivated than plasma BChEs (Heilbronn 1963), especially with AChE-OP complexes, with R-groups of one or two atoms, exclusive of hydrogen or oxygen, such as dimethoxy- and diethoxy-substituted OPs that include the vast majority of agricultural OPs. (3) RBC AChE activity can be corrected, via a hematocrit, to activity per volume of packed RBCs, minimizing fluctuations that can occur with plasma ChE activities due to blood volume changes

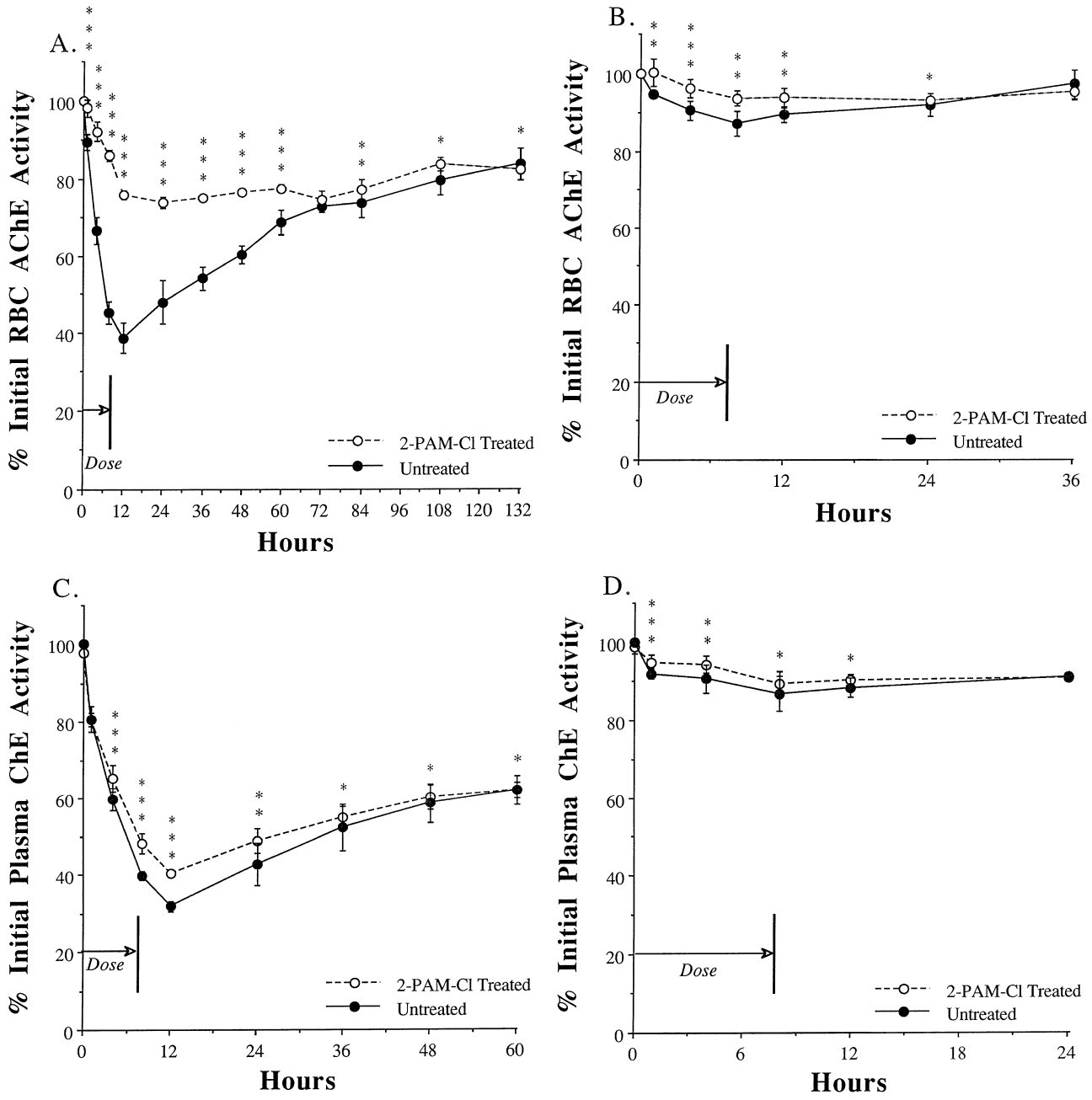

Fig. 2. Reactivation of RBC AChE (A and B) and plasma ChEs (C and D) inhibited by 1 mg/kg dermal ethyl parathion (A and C), parathion (B and D). Rabbits were dermally dosed with either 1 mg/kg of ethyl parathion or 5 mg/kg of methyl parathion. Points are means for three rabbits per treatment, with bars representing standard errors of the mean. Each asterisk indicates statistical significance at ≥5% reactivation ratio and $p \leq 0.05$ from paired Student's *t*-test in one rabbit.

or other factors. (4) Plasma ChE activities may be depressed by a number of factors other than ChE inhibitors, such as liver disease (Silk *et al.* 1979). (5) Finally, (excluding reactivations) OP-inhibited RBC AChE recovers more slowly than plasma ChEs because it is dependent on the relatively slow replacement of new RBCs, approximately 1.4 to 1.5% a day in the rabbit and 0.8% a day in humans (Neuberger and Niven 1951; Lewis *et al.* 1981; George and Abernethy 1983). In the development of the reactivation assay high variability due to loss of AChE activity during removal of the Hb-containing supernatant was overcome by reducing the number of wash steps and sedimenting the RBC ghosts into a 7% sucrose cushion. Care was taken in developing the assay to consider factors important for its optimization, including oxime concentration, reactivation reaction pH, temperature, and rates of aging and spontaneous reactivation (Keijer *et al.* 1974; Moore *et al.* 1995). In general, the higher the oxime concentration, the faster the rate of reactivation and the more aging is inhibited during the reactivation reaction. The optimum reactivation reaction pH was determined using the formula published by Davies and Green (1956). To determine the optimum reactivation pH, the pKa of the oxime (pKa_1; about 8.0 for 2-PAM Cl) and the pKa of the enzyme (pKa_2;

usually about 7.3) are employed in the following equation: $pH_{Opt} = ½(pKa_1 + pKa_2)$. We therefore determined a pH optimum of 7.8 for our system, which is the pH obtained following addition of 10 mM 2-PAM Cl to a 8.0 Reactivation Buffer. Hobbiger (1956) and Amitai *et al.* (1980) also found that a pH around 8.0 is optimum. The temperature optimum was a bit of a compromise, as both oxime and spontaneous reactivation rates increase with increasing temperature (Wilson *et al.* 1992). Thus, we set the reactivation reaction temperature at 25°C. Dimethyl-phosphorylated ChEs tend to have relatively fast rates of aging and spontaneous reactivation (half-lives of 1 to 4 h). Diethyl-phosphorylated ChEs usually have relatively slow rates of aging and spontaneous reactivation (half-lives ranging between 30 to several hundred hours). Di*iso*propyl-phosphorylated ChEs tend to have very rapid rates of aging (half-lives about 4 h) and slow or undetectable rates of spontaneous reactivation (Wilson *et al.* 1992).

Reducing the frequencies of false positives was thought to be more important than reducing false negatives because high exposures to ChE inhibitors would be revealed by the depressed AChE activities of the unreactivated samples. The times necessary for and extent of reactivations achieved were similar to those obtained by Blaber and Creasey (1960), who found that reactivations were successful at least 48 h following subcutaneous injection of TEPP (tetraethylpyrophosphate). The RBC reactivation assay was less effective at reactivating dimethyl-phosphorylated rabbit AChE than diethyl-phosphorylated enzyme, even though the reactivation rate with the former enzyme was greater. This may be due to its greater rates of spontaneous reactivation and aging. Spontaneous reactivation half-lives for various mammalian dimethyl-phosphorylated RBC AChEs vary from 0.85 h in the human (Skrinjaric-Spoljar *et al.* 1973) to 1.4 h in the rabbit (Aldridge 1953; Van Asperen and Dekhuijzen 1958) as compared to 58 h for diethyl-phosphorylated bovine and human RBC AChE (Clothier *et al.* 1981; Wilson *et al.* 1992). Aging rates of dimethyl-phosphorylated RBC AChE ranged from about 3.9 h for humans (Skrinjaric-Spoljar *et al.* 1973) to 8.9 h in bovines (Clothier *et al.* 1981), whereas the respective rates for diethyl phosphorylated RBC AChE ranged from 41 h to 58 h (Heilbronn 1963; Clothier *et al.* 1981; Wilson *et al.* 1992). Blaber and Creasey (1960) were less successful at reactivating dimethyl-phosphorylated sheep RBC AChE than we were with the rabbit enzyme. The ovine enzyme was refractory to oxime reactivation 1 h after intravenous injection of methyl paraoxon. Factors to consider are the differences in the routes of exposure, the degree and rates of OP activation between the two studies, and the lower oxime concentration of 0.25 mM used by Blaber and Creasey (1960). In the experiments reported here, the 100-fold lower oxime concentration used in the plasma than in the RBC reactivations may have been the reason for the lower reactivatability of plasma ChE. If higher oxime concentrations had been used with the plasma assay, additional steps would have been required (*e.g.,* gel filtration) to remove excess oxime to prevent oxime-induced AThCh hydrolysis. To summarize, this method was designed to employ slowly turned over RBC AChE, a high oxime concentration, the reactivator's pH optimum, and a temperature optimum that decreases spontaneous reactivation, while allowing oxime reactivation to proceed. This, coupled to an RBC AChE assay methodology that facilitates hemoglobin removal but reduces concurrent AChE loss during wash steps, makes this a powerful method for documenting OP exposures in mammals. With aged OP-inhibited AChE, which is refractory to reactivation, this method would not yield positive reactivations, but would yield reliable AChE activity depression data, except in cases where spontaneous reactivation was significant. A further disadvantage of our method, unlike the method recently developed by Polhuijs *et al.* (1997) is that even with positive reactivations, it will not provide for identification of the AChE depressing OP.

Acknowledgments. Data herein presented in partial fulfillment of the degree of Master of Sciences, UC Davis, May 1991. Supported in part by the UC Davis Agricultural Health and Safety Center, NIOSH Cooperative Agreement U07/CCU906162-02, UC Davis Center of Environmental Health Sciences, NIEHS ES05707; NIH ES 00202, US Air Force AFOSR-91-0226, the UC Toxic Substances Program and United States Environmental Protection Agency (US EPA) R819658 Center for Ecological Health Research at UC Davis. Although the information in this document has been wholly or in part funded by the US EPA, it may not necessarily reflect the views of the Agency and no official endorsement should be inferred.

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