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Bovine Red Blood Cell Ghost Cholinesterase as a Monitoring Standard

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Monitoring human blood cholinesterase (ChE) activities is becoming increasingly important with the continued worldwide use of anticholinergic pesticides and fears of chemical warfare agents. The availability of standard operating procedures and ChE enzyme preparations are important elements of rapid responses to such life-threatening situations.

Mammalian blood has two main enzymes suitable for detecting the presence of ChE inhibitors. One is commonly referred to as "true" acetylcholinesterase (AChE, EC 3.1.1.7); the other as serum or pseudocholinesterase (BuChE, EC 3.1.1.8). AChEs prefer acetyl substrates, they are inhibited by substrates above 1-2 mM, by 0.5 mM BW284c51 and other selective inhibitors. BuChEs prefer butyryl, sometimes propionyl substrates depending on the species (Wilson, 2001). They are not inhibited by excess substrates at levels below 5-10 mM, and are inhibited by 0.1 mM iso-OMPA. AChE activity is limited to the formed blood elements in the human but not necessarily in other mammals. For example, up to half of the serum ChE activity may be AChE in rodents such as the adult rat and serum AChE may be even higher in the blood of embryos (e.g., fetal calf serum; see Wilson, 2001 for a recent review).

We conducted a comparison test at the request of the EPA with several clinical laboratories on blood ChE of rats exposed to various levels of an organophosphate pesticide (Wilson et al, 1996), we found that the conduct of the assay introduced inaccuracies into the clinical laboratory procedures. For example, not correcting for a transient thiol oxidase activity present in rodent red blood cells (RBC) introduced an indeterminate error in the results. Subsequently, while working with California state agencies and clinical laboratories licensed to monitor farm worker blood ChEs, we found conditions of a commonly used version of the Ellman assay (formerly Boehringer-Mannheim kit, Catalog No. 450035, Boehringer Mannheim Corporation, Indianapolis, IN) decreased the activity of the assay by approximately 40 percent (Wilson et al. 1997). In a comparison project with several clinical laboratories, only two of nine that participated achieved satisfactory results assaying ChE in human blood (Wilson et al. 2002). These findings led to a change in the California state regulations requiring ChE assays be convertible to values obtained from an optimized Ellman assay (Wilson et al.

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2002). As part of a project to help standardize and optimize ChE assays for clinical laboratories, we developed a RBC ghost AChE standard using cows. This brief report describes the RBC ghosts and their enzymatic properties.

MATERIALS AND METHODS

Blood was drawn into heparinized tubes from a dairy herd of Holstein and Guernsey cows of the UC Davis Animal Science department. The blood iced and brought back to the laboratory where RBC ghosts were prepared according to a method modified from Hansen and Wilson (1999) for rabbits. Whole blood was added to a balanced Na-K-phosphate buffer, pH 8.0 and centrifuged for 15 minutes at 2500 xg, 4 °C. The supernatant was removed and the pellet incubated in 20 mOsm sodium phosphate buffer, pH 7.4 to hemolyze the RBCs. A cushion of 7% sucrose (w/v) was carefully added to the centrifuge tube and the preparation centrifuged for 20 minutes at 27,800 xg, 4 °C. The supernatant was removed and the packed RBC membranes solubilized adding 0.5% Ttriton-X-100 in 100 mM sodium phosphate buffer (v/v), pH 7.4.

ChE activities were assayed in triplicate using the standard Ellman assay (Ellman et al. 1961) and a 96 well automated microplate reader similar to that described by Padilla (Padilla et al. 1999). Assay volumes of 320 µl consisted of 250 µl 0.1 M sodium phosphate buffer, pH 8.0, 10 µl of 322 µM dithiobisnitrobenzoate (DTNB), 30 µl of enzyme sample and 30 µl of acetylthiocholine. Acetylthiocholine final concentrations were routinely 1.0 mM; other levels were used as required. Activities were read at 405 nM and 25-27 °C and reported as µmol/min/ml.

RESULTS AND DISCUSSION

The RBC ghost preparation may be stored at –70 °C for a year or more without loss in activity. For example, activity of one preparation kept at –70 °C averaged 0.050 $\pm$ 0.002 (n=13) µmol/min/ml over a period of 368 days stored in a low temperature freezer, but fell off over time in samples kept in the refrigerator (Figure 1). (Standard deviations of nine samples were $\pm$ 3 % of the means, too low to depict in the figure).

The data in Table 1 from 6 cows demonstrate ChE activity was confined to the RBC fraction. This was evidenced by its insensitivity to iso-OMPA and sensitivity to BW 284c51. (Approximately 5% of the enzyme activity was in the plasma fraction, and it, too, was sensitive to BW 284c51.)

Enzyme activities from 20 cows (including the six cows shown in Table 1) led to the same conclusion (Table 2). There was virtually no BuChE activity in whole blood or in the plasma fraction. RBC ghost enzyme activity was run with each experiment, a useful practice with such a standard. The mean activity of 8 trials was 0.896 $\pm$ 0.038 µmoles/min/ml of sample preparation (SEM).

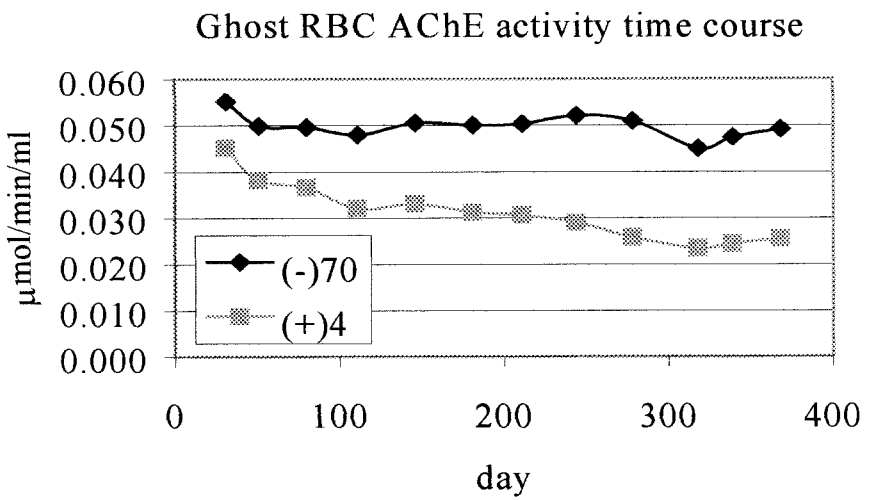

Figure 1. Bovine red blood cell ghost activity with storage. Means of six to nine samples.

Table 1. Acetylthiocholine hydrolysis of bovine blood and its fractions (µmoles/min/ml, mean ± st. dev.)

Treatment	Whole Blood	RBC Fraction	Plasma
None (n=6)	2.23 + 0.59	2.10 + 0.62	0.107 + 0.033
10-4 M iso OMPA	2.17 + 0.53	1.92 + 0.54	0.103 + 0.31
5 x10-5 M BW284c51	-0.28 + 0.01	-0.057 + 0.01	0.0087 + 0.018

Table 2. Acetylthiocholine hydrolysis of 20 bovine blood samples (µmoles/min/ml, mean ± st. dev.)

Fraction	Activity
Whole Blood (No Treatment)	1.98 ± 0.37
With Iso-OMPA	1.95 ± 0.32
Plasma (No Treatment)	0.096 ± 0.031
With Iso-OMPA	0.092 ± 0.032

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Table 3 illustrates the lack of serum BuChE by examining plasma and RBC fractions of one cow with increasing concentrations of acetyl, butyryl and

propionyl thiol substrates. The only appreciable activity present was from RBC acetylthiocholine hydrolysis; it was inhibited by substrate above 2 mM, one of the hallmarks defining "true" AChE.

Table 3. Effect of substrate concentration on ChE activity.

Substrate	µmoles/min/ml	± st. deviation
ACTC	RBC	Plasma
0.1	0.874 ± 0.090	0.052 ± 0.001
0.2	1.340 ± 0.023	0.071 ± 0.001
0.3	1.373 ± 0.164	0.081 ± 0.002
0.5	1.713 ± 0.449	0.092 ± 0.002
1	1.898 ± 0.564	0.098 ± 0.004
2	2.437 ± 0.454	0.092 ± 0.009
3	1.722 ± 0.260	0.108 ± 0.000
5	0.620 ± 0.615	0.109 ± 0.002
BuTC	RBC	Plasma
0.1	-0.047 ± 0.026	0.017 ± 0.001
0.2	-0.046 ± 0.018	0.022 ± 0.00
0.3	-0.083 ± 0.034	0.026 ± 0.001
0.5	-0.052 ± 0.011	0.034 ± 0.001
1	-0.052 ± 0.017	0.093 ± 0.006
2	0.010 ± 0.028	0.057 ± 0.001
3	0.041 ± 0.020	0.069 ± 0.005
5	0.068 ± 0.053	0.077 ± 0.001
PTC	RBC	Plasma
0.1	0.412 ± 0.066	0.033 ± 0.001
0.2	0.605 ± 0.063	0.045 ± 0.001
0.3	0.705 ± 0.019	0.053 ± 0.001
0.5	0.831 ± 0.086	0.063 ± 0.001
1	0.912 ± 0.040	0.076 ± 0.000
2	0.734 ± 0.225	0.077 ± 0.002
3	1.076 ± 0.284	0.080 ± 0.005
5	0.612 ± 0.219	0.091 ± 0.005

(ACTC: Acetylthiocholine; BuTC: butyrylthiocholine; PTC: propionylthiocholine in mM). Activities in µmoles/min/ml, n=3.

Unlike the human, bovine blood had little if any serum ChE activity, whether AChE or BuChE. The lack of serum ChE activity was recognized as early as the 1950s (e.g. Hermenze and Goodwin (1959) in their study of ChE RBC activity in cattle). Nevertheless, Harlin and Ross (1990) used whole bovine blood in a collaborative study of the Ellman assay to recommend a procedure subsequently adopted by the AOAC as a standard. Unfortunately, Harlin and Ross (1990) did not consider that the lack of BuChE in bovine blood may impair its usefulness for

monitoring inhibitors such as chlorpyrifos that prefer serum BuChE (Wilson, 2001).

The ghost preparation maintained activity at –70 °C but declined after a few weeks at 4 °C. Others (e.g. Stefan et al. 1977) found that bovine RBC activity was maintained for "several weeks" or "longer" at –24 to 3 °C. The stability of the RBC ghosts at ordinary freezer temperatures was not investigated here and should be studied. Regardless, the ease with which bovine red blood cell ghosts can be prepared, their stability in storage, as well as the availability of a lyophilized commercial preparation (e.g. Sigma Chemicals Type XII-S Acetylcholinesterase, which is a bovine red blood cell, lyophilized powder) suggests they would be useful as a standard to compare tests between laboratories or within a single laboratory.

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