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Deborah A. Donovan
Western Washington University, deborah.donovan@wwu.edu

Joseph G. Zinkl

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MODIFICATIONS OF A CHOLINESTERASE METHOD FOR DETERMINATION OF ERYTHROCYTE CHOLINESTERASE ACTIVITY IN WILD MAMMALS

Deborah A. Donovan[1] **and Joseph G. Zinkl**[2]
Department of Clinical Pathology, School of Veterinary Medicine, University of California, Davis, California 95616, USA
[1] Current address: Department of Zoology, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z4
[2] To whom correspondence should be addressed.

ABSTRACT: A method to determine erythrocyte cholinesterase (ChE) activity was modified for use in wild mammals. Erythrocyte ChE of California voles (*Microtus californicus*) was primarily acetylcholinesterase (AChE), which was similar to the brain and unlike plasma which was primarily butyrylcholinesterase (BChE). Triplicate erythrocyte AChE analyses from individual animals of several species of wild rodents revealed a mean coefficient of variation of 8.7% (SD = 4.3%). Erythrocyte ChE activity of several wild mammals of California revealed that mule deer (*Odocoileus hemionus*) had the highest erythrocyte AChE activity (1,514.5 mU/ml) and dusky-footed woodrats (*Neotoma fuscipes*) had the lowest activity (524.3 mU/ml). No ChE activity was found in erythrocytes of several species of birds and fish.

Keywords: Acetylcholinesterase, butyrylcholinesterase, erythrocyte, brain, plasma, wild mammals, California voles, deer mice.

INTRODUCTION

Over the past few decades, the use of organophosphate (OP) and carbamate insecticides has increased dramatically. Birds (Zinkl et al., 1979; Hill and Fleming, 1982), fish (Zinkl et al., 1991), and mammals (Jett, 1986) have been adversely affected. Most wildlife poisonings have involved birds, while mammals appear to be less sensitive to anticholinesterase insecticides (Grue et al., 1983). Carbamates and OP's act by binding to the serine active site of acetylcholinesterase (acetylcholine acetylhydrolase: International Union of Biochemistry (IUB) No. 3.1.1.7) (AChE). Acetylcholinesterase is responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) in the cholinergic synapses (Walker and Thompson, 1991). Inhibition of AChE causes ACh to accumulate at the synapse and thus disrupt nerve transmission (Murphy, 1986). In mammals and birds, signs of anticholinesterase poisoning include miosis, excessive salivation, tremors, respiratory difficulty, and tetany (Murphy, 1986). The primary cause of death in vertebrates is respiratory depression due to inhibition of the central respiratory center or the respiratory muscles (Murphy, 1986).

Cholinesterase (ChE) activity is a well established bioassay for diagnosis of OP or carbamate poisoning. Ludke et al. (1975) suggested that 20% depression of brain AChE activity is indicative of exposure in birds and >50% suggests anticholinesterase poisoning as a cause of death. However individual birds can survive depression >50% (Hill and Fleming, 1982). Generally, diagnosis of anticholinesterase poisoning is made by the detection of decreased AChE activity in conjunction with other information such as the presence of pesticide residue on the fur, feathers, ingesta, plants, and water, as well as a history of use of the particular pesticide in the locale of the affected animals (Olson and Christensen, 1980; Hill and Fleming, 1982).

Different tissues may contain different ChE's and thus react differently to anticholinesterases (Witter, 1963). The two main types of ChE's are AChE and butyrylcholinesterase (acylcholine acylhydrolase (IUB No. 3.1.1.8)) (BChE) which is also known as psuedocholinesterase. AChE is found in the nervous tissues of all animals and in the erythrocytes and plasma of many (Walker and Thompson, 1991). Small amounts of BChE are found

in the nervous tissue and in the plasma of most animals. Other nonspecific ChE's are found in tissues (Witter, 1963).

Fleming (1981) suggested that plasma ChE activity is less reliable than brain ChE activity for diagnosing and monitoring anticholinesterase pesticide poisoning since plasma ChE activity exhibits a greater degree of variability among individuals, recovers faster than brain ChE, and may be inhibited to a greater extent without associated clinical signs. O'Brien (1967) also cited the discrepancy between brain and plasma ChE as a major problem in using plasma ChE as a bioassay. Drawbacks in the use of brain ChE activity include not being able to take multiple samples in order to monitor the recovery of an individual as well as being able to use an animal as its own control. Mason and Lewis (1989) suggested that serial measurements on an individual are superior to comparing a ChE activity to a range of normal values due to individual variations in activity. Cholinesterase activity varies with age and physiological status in humans (Lepage et al., 1985), fish (Zinkl et al., 1987), and birds (Gard and Hooper, 1993). It also varies with season (Herbst et al., 1989), nutritional status (Rattner, 1982) and ambient temperature of water for fish (Hogan, 1970), and air temperature for birds (Rattner, 1982). In addition, in cases of poisoning of rare or endangered animals with signs of anticholinesterase insecticide poisoning, a nonlethal method to determine if ChE activity is inhibited is essential (Hooper et al., 1989). It is possible that erythrocyte ChE activity may be a good index of brain ChE activity since both tissues apparently contain primarily AChE (Walker and Thompson, 1991). However, there is great need to develop reliable methods for erythrocyte ChE activity in order to determine how well its activity correlates with that of brain in poisoned animals.

Modifications of the Ellman et al. (1961) technique are the most widely used methods for determining ChE activities. With this method, the rate of hydrolysis of the ACh analog acetylthiocholine (ASCh) (or other thiocholine analog) is determined. Thiocholine produced in the reaction reacts with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and releases 5-thio-2-nitrobenzoic acid which absorbs light in the range of 400 to 420 nanometers (nm) with maximal absorption at 412 nm. Most commonly the AChE activity is determined by the rate of the increased absorbance at 405 nm. This method is acceptably precise (Hawkins and Knittle, 1972). Unfortunately, its use for erythrocytes or whole blood is limited because hemoglobin also absorbs light at similar wavelengths as 5-thio-2-nitrobenzoic acid (Augustinsson et al., 1978). Nevertheless, some investigators have used modifications of the Ellman et al. (1961) technique to determine erythrocyte or whole blood ChE activity (Mason and Lewis, 1989). However, since ChE activity is inhibited in acetylcholinesterase insecticide-poisoning, confidence in erythrocyte or whole blood ChE activity when determined by the Ellman et al. (1961) method is decreased because hemoglobin interference results in a high noise to signal ratio.

Augustinsson et al. (1978) proposed a modification of the Ellman et al. (1961) method for use with erythrocyte hemolysates. The method is based on the reaction of thiocholine with 4,4′-dithiodipyridine (DTDP) to release 4-thiopyridone which absorbs light at 327 nm.

Our objectives were to adapt the method of Augustinsson et al. (1978) for determining the activity of erythrocyte ChE of mammals, birds, and fish; to determine the similarity and differences between brain, plasma and erythrocyte ChE's; and to establish reference values for erythrocyte ChE activity of some wild animals.

MATERIALS AND METHODS

Blood or blood and brain for these studies were obtained from the following California (USA) mammals: mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virgini-*

anus), California sea lion (*Zalophus californianus*), California vole (*Microtus californicus*), brush mouse (*Peromyscus boylii*), deer mouse (*Peromyscus maniculatus*), piñon mouse (*Peromyscus truei*), and dusky-footed woodrat (*Neotoma fuscipes*). The deer were confined to large pens on the University of California, Davis, California (USA); the sea lions were rehabilitated animals from the Marine Mammal Center, Sausalito California. All wild rodents were trapped in two locations (39°43'N, 121°24'W; 39°55'N, 121°30'W) in Butte County, California, as part of a Lyme disease study; some of the California voles were from a colony on the campus.

The methods of Ellman et al. (1961) and Augustinsson et al. (1978) for determining ChE activity were modified as follows: The activities were determined at 25 C in 3 ml of 0.05 M tris buffer (Sigma Chemical Company, St. Louis, Missouri, USA), pH 7.4 in a Beckman DU-68 spectrophotometer (Beckman Instrument Co., Fullerton, California). For the Ellman et al. (1961) technique, 2.5×10^{-4} M 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma Chemical Company), 2.6×10^{-3} M ASCh (or butyrylthiocholine (BSCh)) (Sigma Chemical Company) were used. For the Augustinsson et al. (1978) technique 2.5×10^{-4} M 4,4'-dithiodipyridine (DTDP) (Sigma Chemical Company) was used in place of the DTNB. For some studies 1.0×10^{-5} M eserine (Sigma Chemical Company) or 1.0×10^{-4} M tetraisopropyl pyrophosphoramide (iso-OMPA) (Sigma Chemical Company) were use to inhibit total ChE or BChE, respectively (Fairbrother et al., 1991).

Brain homogenates (1:5) were prepared in 0.05 M tris buffer, pH 8.0. Hemolysates (1:5) were prepared by lysing erythrocytes with distilled water after the erythrocytes had been washed four times with 0.85% saline and the leukocytes had been removed. Plasma was used undiluted. Generally, 0.1 or 0.05 ml of brain homogenate, erythrocyte hemolysate, or plasma was used in the final reaction volume of 3.0 ml. When greater volumes of brain homogenate, erythrocyte hemolysate or plasma were needed in order to increase the rate of the reaction, the amount of tris buffer was reduced in order to maintain a reaction volume of 3.0 ml.

In order to calculate the ChE activities, the molar absorbance coefficient constant at 405 nm for 5-thio-2-nitrobenzoic acid (the product produced from DTNB) of 1.33×10^{4} M^{-1} cm^{-1} was used (Ellman et al., 1961). For 4-thiopyridone (the product of DTDP) the molar absorbance coefficient constant at 327 nm of 1.98×10^{4} M^{-1} cm^{-1} was used (Augustinsson et al., 1978).

In order to assure that the concentrations of ASCh and BSCh were sufficiently high to achieve zero order kinetics, the Michaelis-Menten constants (K_m) and the concentrations necessary to produce maximum activity (V_{max}) or zero order kinetics was determined for brain, plasma and erythrocytes of California voles (York, 1992).

Reproducibility and coefficients of variation of the modified Augustinsson et al. (1978) technique was evaluated on 10 samples from several species analyzed in triplicate (Garber and Carey, 1984).

For the determination of activities in some California mammals, erythrocyte AChE activity was determined by the modified Augustinsson et al. (1978) method. When brain and plasma were available, AChE activities were determined by the Ellman et al. (1961) method.

RESULTS

The absorption spectrum of 5-thio-2-nitrobenzoate and hemoglobin were similar with maximum absorption at about 410 nm (Fig. 1). But the absorption spectrum of 4-thiopyridone was much different that those of hemoglobin and 5-thio-2-nitrobenzoate. The maximum absorbance of 4-thiopyridone was at 327 nm.

The K_m's for ASCh and BSCh were about 1×10^{-4} M (Table 1). The concentrations of 2.6×10^{-3} M used in the assays were sufficiently high to achieve zero order kinetics. The K_m for erythrocyte BSCh was 2×10^{-3} M, which was only slightly lower than the concentration used in the assays. Substrate inhibition was not observed, but the highest concentrations of both ASCh and BSCh were only 1×10^{-2} M.

Brain and plasma AChE activities of California voles determined by both methods were not statistically different (Table 2). It was not possible to perform this study with hemolysate because of the marked hemoglobin absorbance at 405 nm.

Neither brain nor erythrocytes had much activity when BSCh was used as the substrate (Table 3). However, plasma hydrolyzed both BSCh and ASCh, with a slightly greater preference for BSCh. When iso-OMPA was used to inhibit BChE activity, only the plasma activity was significantly inhibited (Table 4).

The mean coefficient of variation for ten erythrocyte hemolysates using the Augus-

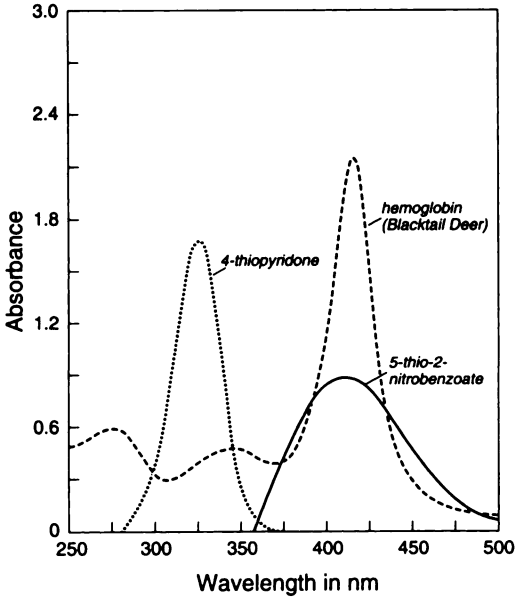

FIGURE 1. Absorbance spectrum of 4-thiopyridone, 5-thio-2 nitrobenzoate and mule deer erythrocyte hemolysate.

tinsson et al. (1978) technique was 8.7% (SD = 4.3), but for some samples the coefficient of variation was greater than 10% (Table 5).

Generally, erythrocyte AChE activities of several mammals varied more than did their brain activities, but the variation was similar to that of the serum (Table 6). No erythrocyte ChE activity was found in several species of birds or fish (data not shown).

DISCUSSION

In principle the ChE technique proposed by Augustinsson et al. (1978) is very similar to that of Ellman et al. (1961). Both rely on the formation of thiocholine which in turn reacts with a chromogen to form a colored product. The rate of formation of this product is proportional to the ChE activity. The difference between the two methods is the chromogen used and consequently the colored product formed. The Ellman et al. (1961) method uses DTNB and forms 5-thio-2-nitrobenzoate which can be detected by its absorption at 405 nm. Augustinsson et al. (1978) substituted DTDP for DTNB; the DTDP reacts with thiocholine to form 4-thiopyridone which absorbs maximally at 327 nm. The Ellman et al. (1961) technique is difficult to use for analysis of erythrocyte ChE because 5-thio-2-nitrobenzoate has a similar absorption spectrum as hemoglobin (Fig. 1). Because of this, erythrocyte hemolysates must be diluted greatly to overcome the high hemoglobin absorption in order to be within the sensitive range of most spectrophotometers. Because the samples must be diluted greatly, it is very difficult to accurately detect meaningful changes in absorption with hemolysates using the Ellman et al. (1961) method. Spectral interference by hemoglobin does not occur with the Augustinsson et al. (1978) method because hemoglobin and 4-thiopyridone absorb at very different wavelengths (Fig. 1). As a consequence, it is possible to use a more concentrated hemolysate in order to determine AChE activity. The ability to use a more concentrated hemolysate is of particular importance when ChE activity is quite low as could occur in animals poisoned with anticholinesterase insecticides.

TABLE 1. Michaelis-Menten constants (K_m) for the substrates acetylthiocholine (ASCh) and butyrylthiocholine (BSCh) for cholinesterase of erythrocytes, brain, and plasma of California voles.

Tissue[a]	K_m	
	ASCh	BSCh
Brain	1.39×10^{-4} M	6.15×10^{-4} M
Erythrocyte	8.55×10^{-5} M	2.04×10^{-3} M
Plasma	1.22×10^{-4} M	9.82×10^{-5} M

[a] Tissues from five animals were used and the K_m was determined in triplicate.

TABLE 2. Mean (±SD) brain and plasma cholinesterase activities of four California voles determined by modifications of Augustinsson et al. (1978) and Ellman et al. (1961) techniques.

Tissue	Method	
	Augustinsson et al.	Ellman et al.
Brain (mU/mg)	2.44 ± 0.98	2.50 ± 0.74
Plasma (mU/ml)	1,284.3 ± 249.9	1,242.5 ± 231.5

TABLE 3. Specificity for the substrates acetylthiocholine (ASCh) and butyrylthiocholine (BSCh) for erythrocyte, plasma, and brain cholinesterase of California voles.

Tissue	Mean (±SD) activity	
	ASCh substrate	BSCh substrate
Brain (n = 5) (mU/mg)	5.33 ± 0.76	0.23 ± 0.14
Erythrocyte (n = 5) (mU/ml)	1,162.0 ± 91.9	221.6 ± 73.2
Plasma (n = 4) (mU/ml)	1,749.9 ± 308.5	2,487.7 ± 644.8

Based on the K_m of ASCh for hemolysates, plasma and brain ChE and the K_m of BSCh and for plasma and brain as well as the V_{max} using ASCh and BSCh, we believe that substrate concentrations of 2.6×10^{-3} M used in the assays were sufficient to achieve zero order kinetics for most of the tissues and substrates (Table 1). The K_m of BSCh for erythrocyte ChE was 2×10^{-3} M; this was greater than that of plasma and brain. Thus, 2.6×10^{-3} M BSCh may not have been sufficient to produce zero order kinetics for erythrocyte cholinesterase. However considerable error could have occurred in determining the erythrocyte K_m for BSCh because the activity using BSCh was very low. Butyrylthiocholine was not considered an appropriate substrate for determination of erythrocyte activity because erythrocyte BChE activity was very low (Table 3).

For tissues in which no hemoglobin interference occurred, both techniques produced similar results (Table 2). Furthermore, similar results using both methods have been obtained using ASCh, propionylthiocholine and BSCh for brain and plasma (or serum) of several domestic animals (Zinkl, unpubl.).

Based on these studies, it appears that erythrocyte ChE activity was primarily attributable to AChE. This was similar to the brain activity and quite different than plasma ChE. Neither erythrocytes nor brain had much activity when BSCh was used as a substrate, but plasma had considerable activity (Table 3). All three tissues had high activity with ASCh. In addition, plasma ChE was markedly inhibited by iso-OMPA, but erythrocyte and brain ChE's were not (Table 4). Since iso-OMPA is a specific inhibitor of BChE (Fairbrother et al., 1991), it was apparent that erythrocyte and brain ChE's are primarily AChE, and plasma ChE is a mixture of AChE and BChE.

The within sample variation or precision for erythrocyte AChE (Table 5) was acceptable and similar to that found in our laboratory for the Ellman et al. (1961) technique for brain and plasma (Zinkl, unpubl.).

Erythrocyte AChE activity varied markedly within the limited numbers of mammalian species sampled (Table 6). Thus it is necessary to establish baseline values when other species are sampled. Because there are many factors that can affect ChE activities we believe that investigators should attempt to establish baseline

TABLE 4. Inhibition by tetraisopropyl pyrophosphoramide (iso-OMPA) of erythrocyte, plasma, and brain cholinesterase of California voles.

Tissue	Mean (±SD) activity	
	Substrate	Substrate and iso-OMPA
Brain (n = 4) (mU/mg)	5.27 ± 0.86	5.28 ± 1.03
Erythrocyte (n = 4) (mU/ml)	1,008.7 ± 99.6	1,072.3 ± 232.6
Plasma (n = 3) (mU/ml)	2,791.1 ± 998.3	27.0 ± 7.8

TABLE 5. Erythrocyte acetylcholinesterase activities for triplicate trials on ten individual animals.

Species	Mean activity ± 1 SD mU/ml	Range[a]	Coefficient of variation %
Deer mouse	616.1 ± 98.5	195.3	16.0
Brush mouse	591.0 ± 89.6	172.8	15.2
Brush mouse	844.0 ± 30.4	60.1	3.6
Brush mouse	1,039.3 ± 76.8	142.8	7.4
Brush mouse	1,207.1 ± 74.1	135.3	6.1
Brush mouse	966.7 ± 86.4	172.8	8.9
Piñon mouse	1,154.5 ± 89.0	157.8	7.7
Piñon mouse	946.7 ± 118.1	217.9	12.5
California vole	1,126.9 ± 74.0	142.7	6.6
California vole	1,642.9 ± 50.0	112.7	3.0
Mean (±SD)			8.7 (4.3)

[a] Difference between maximum activity and minimum activity.

values whenever they are investigating possible OP or carbamate poisoning. Erythrocyte ChE activity has the potential to allow an animal to act as its own control because, like brain ChE activity, it is primarily AChE; it also is possible to sample the same animal several times. Studies using the Ellman et al. (1961) method for brain and plasma ChE activities and the Augustinsson et al. (1978) method for erythrocyte AChE are needed to determine how well the ChE activities of plasma and erythrocytes of animals exposed to anticholinesterases correlate with brain ChE activity.

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TABLE 6. Cholinesterase activities of some wild mammals of California.

Species	Mean (±SD) activity (sample size) Erythrocyte mU/ml[a]	Plasma mU/ml[b]	Brain mU/mg[b]
Deer mouse	967.9 ± 320.3 (9)	ND[c]	17.71 ± 3.33 (3)
Brush mouse	921.0 ± 255.5 (21)	ND	10.25 ± 0.43 (3)
Piñon mouse	839.4 ± 184.5 (21)	ND	10.27 ± 0.73 (13)
California vole	1,041.8 ± 228.7 (16)	1,604.4 ± 511.8 (12)	5.64 ± 1.07 (11)
Dusky-footed woodrat	524.3 ± 131.1 (7)	ND	3.48 ± 0.11 (2)
Mule deer	1,514.5 ± 808.2 (5)	143.0 ± 63.6 (5)	ND
White-tailed deer	1,016.5 ± 200.5 (4)	ND	ND
California sea lion	588.8 ± 284.1 (3)	753.9 ± 168.6 (3)	3.47 ± 2.75 (2)

[a] Determined by the Augustinsson et al. (1978) technique.
[b] Determined by the Ellman et al. (1961) technique.
[c] ND, not determined.

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