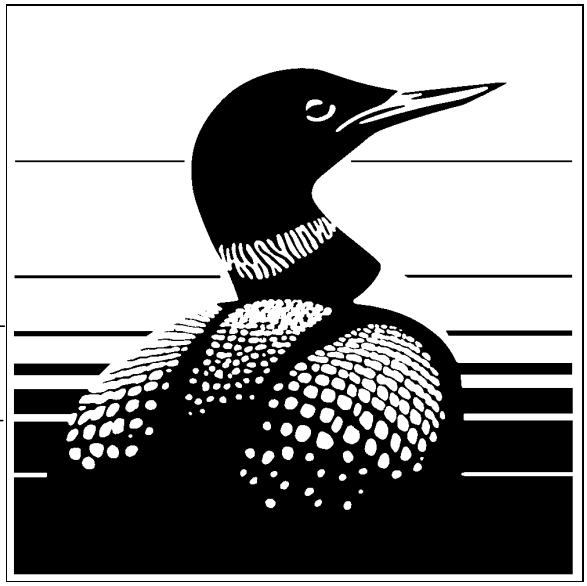
Biochemical Methods to Determine Cholinesterase Activity in Wildlife Exposed to Pesticides

S. Trudeau and G. Sans Cartier

National Wildlife Research Centre 2000
Canadian Wildlife Service
Environmental Conservation Branch



Technical Report Series Number 338

Environment Canada | Environnement Canada

Canadian Wildlife Service | Service canadien de la faune

Canada

TECHNICAL REPORT SERIES
CANADIAN WILDLIFE SERVICE

This series of reports, established in 1986, contains technical and scientific information from projects of the Canadian Wildlife Service. The reports are intended to make available material that either is of interest to a limited audience or is too extensive to be accommodated in scientific journals or in existing CWS series.

Demand for these Technical Reports is usually confined to specialists in the fields concerned. Consequently, they are produced regionally and in small quantities; they can be obtained only from the address given on the back of the title page. However, they are numbered nationally. The recommended citation appears on the title page.

Technical Reports are available in CWS libraries and are listed in the catalogue of the National Library of Canada in scientific libraries across Canada. They are printed in the official language chosen by the author to meet the language preference of the likely audience, with a résumé in the second official language. **To determine whether there is significant demand for making the reports available in the second official language, CWS invites users to specify their official language preference. Requests for Technical Reports in the second official language should be sent to the address on the back of the title page.**

SÉRIE DE RAPPORTS TECHNIQUES
DU SERVICE CANADIEN DE LA FAUNE

Cette série de rapports donnant des informations scientifiques et techniques sur les projets du Service canadien de la faune (SCF) a démarré en 1986. L'objet de ces rapports est de promouvoir la diffusion d'études s'adressant à un public restreint ou trop volumineuses pour paraître dans une revue scientifique ou l'une des séries du SCF.

Ordinairement, seuls les spécialistes des sujets traités demandent ces rapports techniques. Ces documents ne sont donc produits qu'à l'échelon régional et en quantités limitées; ils ne peuvent être obtenus qu'à l'adresse figurant au dos de la page titre. Cependant, leur numérotage est effectué à l'échelle nationale. La citation recommandée apparaît à la page titre.

Ces rapports se trouvent dans les bibliothèques du SCF et figurent aussi dans la liste de la Bibliothèque nationale du Canada utilisée dans les principales bibliothèques scientifiques du Canada. Ils sont publiés dans la langue officielle choisie par l'auteur en fonction du public visé, avec un résumé dans la deuxième langue officielle. **En vue de déterminer si la demande est suffisamment importante pour produire ces rapports dans la deuxième langue officielle, le SCF invite les usagers à lui indiquer leur langue officielle préférée. Il faut envoyer les demandes de rapports techniques dans la deuxième langue officielle à l'adresse indiquée au verso de la page titre.**

BIOCHEMICAL METHODS TO DETERMINE CHOLINESTERASE ACTIVITY IN WILDLIFE EXPOSED TO PESTICIDES

S. Trudeau and G. Sans Cartier

National Wildlife Research Centre
Canadian Wildlife Service
Environment Canada

Technical Report Series No. 338
Headquarters 2000
Canadian Wildlife Service

Issued under the Authority of the
Minister of Environment
Canadian Wildlife Service

Catalogue No. CW69-5/338E
ISBN 0-662-28025-3

Copies may be obtained from:

Canadian Wildlife Service
National Wildlife Research Centre
100 Gamelin Blvd.
Hull, Québec, Canada
K1A 0H3

This report may be cited as:

Trudeau, S. and Sans Cartier, G. (2000) Biochemical Methods to Determine Cholinesterase Activity in Wildlife Exposed to Pesticides. Technical Report Series No. 338. Canadian Wildlife Service, Headquarters, Hull, Québec, Canada.

PREFACE

The Biomarker Laboratory (Laboratory Services Section) of the National Wildlife Research Centre has been providing cholinesterase (ChE) activity measurements in wildlife tissues in support to the National Wildlife Toxicology Program since the early 1990s. This report contains detailed descriptions of the analytical methodologies used, which are based on well established spectrophotometric techniques. It has been prepared, more specifically, to provide a citable reference for our clients and collaborators.

Three methods are included. They have been written in a format consistent with the CAN/CSA-Z-753-94 standard (Guidelines for the Documentation of Test Methods, Canadian Standards Association), which in turn is based on recommendations described by the International Standards Organization (ISO) Guide 25. Method ***MET-BMK-CHE-01*** is used for the analysis of plasma samples and brain homogenates with acetylthiocholine iodide as substrate whereas ***MET-BMK-CHE-02*** is used with whole blood samples using propionylthiocholine iodide. Method ***MET-BMK-CHE-03*** is used in conjunction with MET-BMK-CHE-01 for the differential diagnosis of organophosphorus and carbamate insecticides poisoning. It should be noted that some sections of the methods somewhat overlap and internal referencing is therefore used to minimize redundancy.

Standard Operating Procedures (SOPs) specific to our organization are cited throughout this document. These SOPs are not readily available in the published literature but can be obtained from the authors, upon request.

The assay parameters presented in the methods are used in the context of wildlife exposure to ChE inhibiting substances. A thorough validation of these parameters should be made before using these methods to insure that they are applicable to other studies. The names of manufacturers, suppliers and trade names are included only to document the exact assay conditions adopted by the Biomarker Laboratory. Other equivalent products, instruments or reagents from other sources may also give satisfactory results.

PRÉFACE

Méthodes biochimiques pour déterminer l'activité de la cholinestérase chez les animaux sauvages exposés aux pesticides.
Depuis le début des années 1990, le Laboratoire de dépistage biologique de la Section des services de laboratoire du Centre national de la recherche faunique fournit, dans le cadre de son Programme national de surveillance des effets des produits toxiques sur les espèces sauvages, un service d'analyse de cholinestérase. Ce rapport a été écrit dans le but spécifique de fournir une référence pouvant être citée par nos clients et collaborateurs. Il décrit avec détails les méthodes analytiques utilisées, dont le principe découle de techniques spectrométriques bien établies.

On y retrouve une compilation de trois méthodes analytiques, rédigées selon le format proposé dans la norme CAN/CSA-Z753-94 («*Guidelines for the Documentation of Test Methods*», Association canadienne de normalisation), en accord avec le Guide 25 de l'Organisation internationale de normalisation (ISO). La méthode ***MET-BMK-CHE-01*** est utilisée pour l'analyse du plasma et d'homogénats de cerveau avec l'acétylthiocholine iodide comme substrat tandis que la méthode ***MET-BMK-CHE-02*** est utilisée pour l'analyse du sang avec le propionylthiocholine iodide. La méthode ***MET-BMK-CHE-03*** est utilisée de pairs avec la MET-BMK-CHE-01 pour faire la distinction entre un empoisonnement par un composé organophosphoré ou un carbamate. Il est à noter que certaines sections des méthodes se chevauchent et, afin d'éviter les répétitions, des renvois internes sont utilisés.

Tout au long du document on fait référence à des modes opératoires normalisés («*SOPs*») qui sont spécifiques à notre organisation. Ces procédures ne sont pas disponibles dans la littérature mais peuvent être obtenues en communiquant directement avec les auteurs.

Les paramètres d'analyse présentés dans ces méthodes sont utilisés dans le contexte de l'exposition d'animaux sauvages à des substances inhibitrices de la cholinestérase. Une validation approfondie de ces paramètres devrait être faite avant d'utiliser ces méthodes pour d'autres études. Le nom des manufacturiers, fournisseurs et nom de commerce des produits sont inclus uniquement dans le but de documenter les conditions d'analyse précises utilisées par le Laboratoire de dépistage biologique. Des produits, instruments ou réactifs équivalents provenant d'autres sources peuvent aussi donner des résultats satisfaisants.

TABLE OF CONTENTS

SPECTROPHOTOMETRIC DETERMINATION OF CHOLINESTERASE ACTIVITY IN PLASMA AND BRAIN

based on Ellman's method [2.1] as modified by Hill and Fleming [2.2]

1. SCOPE AND FIELD OF APPLICATION

This is a colorimetric method for measurement of cholinesterase (ChE) activity in plasma and brain. It is used in wildlife toxicology research to detect exposure to cholinesterase-inhibiting pesticides (organophosphorus and carbamates). A depression in plasma or brain ChE activity below the normal levels of the species is often used as evidence of exposure and, in the case of field mortalities, the cause of death [2.3]. The minimum detectable amount of this method has been estimated to be around 50 µmol/min/L for plasma sample and 0.2 µmol/min/g for brain tissue.

2. REFERENCES

2.1. Ellman, G.L., Courtney, K.D., Andres, V. Jr. and Featherstone, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmac.*, 7, 88-95.

2.2. Hill, E.F. and Fleming, W.J. (1982) Anticholinesterase poisoning of birds: Field monitoring and diagnosis of acute poisoning. *Environmental Toxicology*, 1(1), 27-38.

2.3. Ludke, J.L., Hill, E.W. and Dieter, M.P. (1975) Cholinesterase (ChE) response and related mortality among birds fed ChE inhibitors. *Arch. Environ. Contam. Toxicol.*, 3, 1-21.

2.4. Lawson, A.A. and Barr, R.D. (1987) Acetylcholinesterase in red blood cells. *American Journal of Hematology*, 26, 101-112.

2.5. Walker, C.H. and Thompson, H.M. (1991) "Phylogenetic distribution of cholinesterases and related esterases". In Mineau, P. [Ed.] Cholinesterase-inhibiting Insecticides - Their Impact on Wildlife and the Environment - Chemicals in Agriculture, Vol. 2, Elsevier, 1-18.

2.6. Boehringer Mannheim. Cholinesterase Test-combination sheet and Precinorm E control sera sheet. Nov '84.

2.7. Hart, A.D.M. and Westlake, D.E. (1986) Acetylcholinesterase activity and effects of Chlorfenvinphos in regions of the starling brain. *Bul. Envi. Conta. Toxicol.*, 37, 774-782.

2.8. Peakall D. (1992) "Biomarkers of the nervous system" in Animal Biomarkers as Pollution Indicators, Chapman & Hall, Ecotoxicology Series 1, 19-45.

2.9. Walmsley, T.A., Abernethy, M.H. and Fitzgerald, H.P. (1987) Effect of daylight on the reaction of thiols with Ellman's reagent, 5,5'-Dithiobis (2-Nitrobenzoic acid), *Clin. Chem.* 33(10), 1928-1931.

2.10. Marden, B.T., Fairbrother, A. and Bennett, J.K. (1994) Interlaboratory comparison of cholinesterase assay measurements, *Environ. Toxic. and Chem.*, 13(11), 1761-1768.

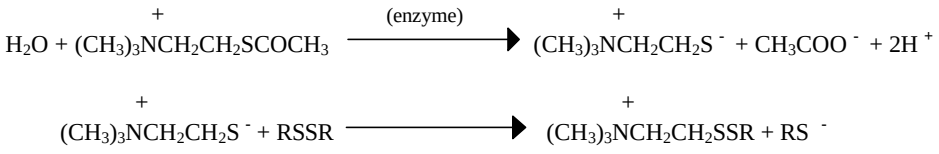
2.11. Fairbrother, A., Marden, B.T., Bennett, J.K. and Hooper, M.J., "Methods used in determination of cholinesterase activity". In Mineau, P. [Ed.] Cholinesterase-inhibiting Insecticides - Their Impact on Wildlife and the Environment - Chemicals in Agriculture, Vol. 2, Elsevier, 35-71.

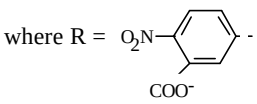
3. PRINCIPLES AND DEFINITIONS

Esterases that hydrolyze choline esters at a higher rate than other esters have been designated as cholinesterases (ChEs). There are two major types based on substrate specificities and susceptibility to inhibitors. First, acetylcholinesterase (EC 3.1.1.7) [a], also known as "red cell", "true", "specific" or "type I" cholinesterase, catalyses the hydrolysis of the neurotransmitter acetylcholine to choline and acetic acid. The enzyme exists primarily in nerve cells involving cholinergic synaptic transmission, but is also found in a variety of other neuronal and non-neuronal cells. It is subject to high substrate inhibition. The second is plasma butyrylcholinesterase (EC 3.1.1.8) also known as "acylcholine acyl hydrolase", "nonspecific", "pseudo", or "type II" cholinesterase. It is found principally in plasma and it acts on a variety of choline, phenyl, nitrophenyl and other types of esters [2.4].

In avian species, the activity measured in brain is largely due to acetylcholinesterase whereas in the plasma, various enzymes are involved [2.5] and the relative proportion of these enzymes varies between species. This method does not distinguish between the enzymes and the term "ChE activity" refers to the combined activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). In brain samples, the activity is reported as AChE activity.

Cholinesterase hydrolyzes acetylthiocholine iodide (AThChI) into thiocholine and acetate. Thiocholine reacts with dithiodinitrobenzoic acid (DTNB) to form thionitrobenzoic acid which is measured spectrophotometrically at 405 nm. The rate at which it is formed represents ChE activity [2.1]. The reaction is:





[a] Enzyme Commission classification

4. REAGENTS, SOLUTIONS, MATERIALS AND STANDARDS

SAFETY PRECAUTIONS

⇒ General safety rules and waste disposal procedures that apply to the Biomarker Laboratory must be followed (ref. Laboratory safety binder).

⇒ Adequate protective equipment must be used: lab coat, gloves, mask (when preparing solutions and tissues), cryogloves and face shield (when retrieving samples stored in liquid nitrogen).

⇒ Material Safety Data Sheets (MSDS) for the products used in the assay must be read.

4.1. Reagents

4.1.1. Sodium phosphate dibasic, Na_2HPO_4, M.W. 141.96, Fisher S-374

4.1.2. Potassium phosphate monobasic, KH_2PO_4, M.W. 136.09, Fisher P-285

4.1.3. 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), crystalline, M.W. 396.40, Sigma D-8130

4.1.4. Acetylthiocholine iodide (AThChI), crystalline, M.W. 289.20, Sigma A-5751

4.1.5. Triton X-100, BDH R06433

4.1.6. Purified water from the Milli-RO / Milli-Q system.

4.2. Solutions

4.2.1. *Sodium phosphate 0.05 M* - Dissolve 7.1 g Na_2HPO_4 in water and dilute to 1L.

4.2.2. *Potassium phosphate 0.05 M* - Dissolve 0.68 g KH_2PO_4 in water and dilute to 100 mL.

4.2.3. *Phosphate buffer 0.05 M, pH 7.9* (at room temperature) - Add 68 mL potassium phosphate solution to 932 mL of sodium phosphate solution. Adjust pH, if necessary, with H_3PO_4 or base.

4.2.4. *DTNB, $2.5x10^{-4}$ M in buffer pH 7.9* - Dissolve 49.5 mg DTNB in phosphate buffer pH 7.9 and dilute to 500 mL. Transfer immediately into an amber bottle and refrigerate. - ***Shelf life: 3-4 weeks at 4°C***

4.2.5. *Acetylthiocholine iodide (AThChI) 0.156 M* - Dissolve 225 mg AThChI in water and dilute to 5 mL to have a final substrate concentration of $5x10^{-3}$ M. (or 45 mg in 5 mL to have a final substrate concentration of $1x10^{-3}$M - see Section 11) - ***Shelf life: prepare fresh daily***

4.2.6. *Triton X-100 (1% v/v) in buffer* - Add 1 mL Triton X-100 to 99 mL of phosphate buffer pH 7.9. (Prepare in a beaker and gently mix with the magnetic stirrer to avoid foaming).

4.3. Control Serum

Open a bottle of Precinorm E (Boehringer Mannheim 125113) and add exactly 3 mL of purified water. Close bottle carefully and let stand for 5 min. Dissolve contents completely by swirling gently, avoiding the formation of foam. Do not shake. Freeze the remaining reconstituted serum as ca 200 µL aliquots in labeled plastic microcentrifuge tubes at -70°C (to be used in subsequent assays; it is stable for at least 1 month at -20°C [2.6]). ***Note:*** This quality control material is used with plasma and brain specimen (see Section 10.1).

5. AUXILIARY EQUIPMENT

5.1. Glassware and Labware

5.1.1. Pipets (Biohit 5000 or Rainin and Gilson Pipetman 100 µL)

5.1.2. Glass fixed-needle Hamilton syringe, 25 µL

5.1.3. Disposable transfer pipets

5.1.4. Disposable acrylic cuvettes, 1 cm lightpath (Sarstedt 67.738)

5.1.5. Volumetric flasks, 100 and 500 mL, and 1 L

5.1.6. Graduated glass tube, 5 mL

5.1.7. Beakers, 250 mL and 1 L

5.1.8. Dispensing bottle (for purified water)

5.1.9. Ice bucket

5.1.10. Tubes, microcentrifuge

5.1.11. Cryovials, 1.0 or 1.8 mL

5.2. Equipment

5.2.1. Temperature probe (pH meter - Hanna instruments HI8417)

5.2.2. pH meter (PerpHect Meter, model 350, VWR)

5.2.3. Analytical balance (Sartorius RC210P) and top-loading balance (Mettler PM4000)

5.2.4. Water bath (Precision Instrument) to preincubate the cuvettes

5.2.5. Vortex mixer

5.2.6. Magnetic stirrer and magnetic bar

5.2.7. Tissue homogenizer (Heidolph RZR-2000) with Wheaton tubes and pestles - for brain tissues

5.3. Instrumentation

5.3.1. Spectrophotometer (Hewlett-Packard Diode Array, model 8452A) with a thermostated cell holder

5.3.2. Computer with HP89530A MS™-DOS UV/VIS operating software, version 1.0 and printer

5.3.3. Lauda circulator bath (Brinkmann, model MS-3) - to control the temperature of the spectrophotometer cell holder

5.3.4. Centrifuge (Beckman JA-21) with various rotors and centrifugation tubes - for brain tissues

6. SPECIMEN OR SAMPLE HANDLING REQUIREMENTS

Blood samples should be centrifuged as soon as possible after collection and the plasma should be kept cold until the analysis. Pending centrifugation, the blood must not be frozen to prevent haemolysis. If the analysis is not performed shortly after the collection, it is advisable to freeze the samples (plasma, brain tissue or whole head) in a freezer capable of maintaining -70°C (or lower). Long-term storage should be avoided in order to minimize problems associated with dehydration of the samples and stability of inhibited enzymes.

7. PROCEDURE

7.1. Sample Preparation

7.1.1. *Brain tissue*

7.1.1.1. Refrigerate the centrifuge and rotor (ref. step 7.1.1.6) at 4°C.

7.1.1.2. If the brain is still in the cranium, refer to SOP-BMK-PROC-06 (***Appendix A***) for details concerning dissection. *Note*: the brain is removed completely because the enzyme is not uniformly distributed in the brain [2.7]. If it is necessary to reduce sample size or if not all the sample is available for the assay, use **medially** bisected section. The right section is then being used for the analysis and the left lobe is transferred in a storage container and frozen at -70°C.

7.1.1.3. Place a Wheaton tube of the appropriate size in a beaker with ice on the top-loading balance. For tissues weighing 1 g or less use 2.5 mL tube; for samples weighing 1 to 2.5 g use 10 mL tube; for samples weighing 2.5 to 4 g use 15 mL tubes and for samples weighing 4 to 17 g use the 55 mL or 70 mL tube.

7.1.1.4. Transfer the partially thawed brain into the Wheaton tube and record its weight on the "Sample preparation" form (FORM-BMK-01). For small brains, i.e., weighing less than 400-500 mg, the analytical balance should be utilized.

7.1.1.5. Using a tissue grinder, homogenize **thoroughly** with 3 volumes of ice cold 1% Triton X-100 at a ratio of 250 mg/mL (e.g., if brain weight is 3 g, add 9 mL). Homogenization is done to approximately the same degree for each sample by moving the tube up and down (ca 20 times) and twisting a few strokes each time, keeping the grinding mortar on ice. *Note*: homogenizer setting: ca 600 RPM.

7.1.1.6. Prepare the other samples the same way, then transfer into centrifuge tubes of appropriate size (ref. table below). Insure that the tubes are well equilibrated in the rotor and centrifuge at 17 000 g during 45 minutes at 4°C.

Rotor number	Centrifuge tube size (mL)	Rotor speed (RPM)
JA-18.1	1.8	12 000
JA-21	15	14 500
JA-18	35 & 50	12 500

7.1.1.7. Slowly decant the supernatant into a labeled test tube. Gently mix by inversion and transfer a 1 mL aliquot into a labeled Nunc vial for storage at -70°C. Keep the rest of the sample on ice until assayed as described in Section 7.3.

- Minimum sample size recommended : 100 mg of tissue. When the brain is too small the assay is done without centrifuging the homogenate.

7.1.2. *Plasma samples*

No sample preparation is required for the plasma; keep on ice at all times and note the condition of the sample (lipemic, haemolyzed, etc.) on the form FORM-BMK-13a or 13b.

- Minimum sample size recommended : 60 µL (or 250 µL if reactivation is to be done).

7.2. Instrument Operating Parameters

- **Computer file path :** C:\HP8452
- **Mode :** F3 - Kinetic
- **Wavelength :** F1 - Single Wavelength (406,0)

  Note: 406 nm is used instead of 405 because the detector has 2 nm resolution.
- **Run Time :** F3 - RUN = 120 (readings from 60 sec to 120 sec with readings every 15 sec)
- **Stabilization Time:** F3 - START = 60 sec
- **Cycle and Integration Time :** F5 - CYCLE = 15 sec and INTGR = 5
- **Print format :** F2 - Tabular

7.3. Analysis

7.3.1. Turn on the spectrophotometer about 1 hour prior to the assay (period required to warm up the lamp).

7.3.2. Turn on the computer and enter the instrument operating parameters as described in Section 7.2.

7.3.3. Turn on the Lauda Circulator water bath, in order to maintain the cuvette temperature at 30°C, and the water bath used to pre-incubate the cuvettes.

7.3.4. Dispense 3 mL of DTNB/buffer solution (chromogen) in cuvette with the Rainin or Biohit pipet and pre-incubate in water bath or directly in the spectrophotometer unit. Insert the temperature probe in the cuvette.

7.3.5. When the temperature has reached 27°C, add 20 µL of thoroughly mixed brain homogenate (or plasma) using a 25 µL Hamilton syringe and gently mix the solutions with the probe. *Note:* for samples having high activity it may be necessary to use only 10 µL of brain homogenate to insure that the substrate is in excess at all times.

7.3.6. When the temperature is at 30.0 ± 0.2°C, remove the probe and press F8 - "SCAN BLANK" to zero the spectrophotometer at 406 nm. Then press F10 to exit the menu and F9 - "SCAN SCREEN". Add 100 µL of substrate solution (acetylthiocholine iodide) with the 100 µL Pipetman pipet and start the scan by pressing F1 - "BEGIN SCAN".

7.3.7. Immediately mix the solutions using a disposable transfer pipet for about 15 sec and then allow sample to stabilize. The change in absorbance will be recorded for 1 min starting 60 sec after the addition of the substrate, as specified in the instrument parameters.

7.3.8. Once the scan is completed, press F8 and enter the sample name.

7.3.9. Press F7 to access the calculation screen. Press F6 and enter the appropriate conversion factor (ref. Section 8.3) and F7 to specify the results units. Finally press F1 to obtain the calculated results.

7.3.10. Print out the results by pressing F10 and F9. Then Press F10 to return to the screen “kinetic mode” to blank scan another sample.

7.3.11. Report the ChE activity measured on the worksheet (FORM-BMK-13a or 13b) and subtract the value of the spontaneous hydrolysis if applicable (ref. Section 8.4).

8. EXPRESSION OF RESULTS

8.1. Plasma

The rate of enzyme activity is reported in µmole of substrate hydrolyzed per minute per litre at 30°C. It is calculated by multiplying the change in absorbance per minute for the linear portion of the reaction by a factor of 11 700.

$$\text{Activity} = \frac{\Delta A/\text{min} \times \text{assay volume (mL)} \times 1\,000}{\text{abs. coeff.} \times \text{lightpath (cm)} \times \text{sample vol. (mL)}}$$

where: ΔA = change in absorbance
1 000 = to convert from units/mL to units/L
abs. coeff. = absorbance coefficient for thionitrobenzoic acid at 405 nm.

for a total volume of 3.12 mL and a 1 cm lightpath the calculation is:

$$\frac{\Delta A/\text{min} \times 3.12\ \text{mL} \times 1\,000}{13.33\ \text{cm/µmol} \times 1\ \text{cm} \times .02\ \text{mL}} = \Delta A/\text{min} \times 11\,700 = \text{µmol/min/L}$$

8.2. Brain

The rate of enzyme activity is reported in µmole of substrate hydrolyzed per minute per gram of tissue (wet weight) at 30°C; it is calculated by multiplying the change in absorbance per minute by 46.8.

$$\text{Activity} = \frac{\Delta A/\text{min} \times 3.12\ \text{mL} \times 1\,000}{13.33\ \text{cm/µmol} \times 1\ \text{cm} \times .02\ \text{mL} \times 250\ \text{mg/mL}} = \Delta A/\text{min} \times 46.8 = \text{µmol/min/g}$$

8.3. Conversion Factors

Calculations are done directly by the UV/VIS software but since absorbance change per second are given, the above factors have to be multiplied by 60. The factors will then be:

- for 20 µL plasma samples : 702 000
- for 20 µL brain samples (containing 250 mg tissue/mL) : 2 808
- for 10 µL brain samples (containing 250 mg tissue/mL) : 5 616

8.4. Spontaneous Hydrolysis of the Substrate

Reconstituted acetylthiocholine iodide (AThChI) yields reduced rate of apparent ChE activity because of non enzymatic hydrolysis. This value is determined at the beginning of each working day by adding AThChI directly to DTNB and measuring the change in absorbance in the absence of ChE enzyme and is subtracted from the total hydrolysis value obtained. With the assay conditions of the present method, the ΔA/min is around 0.0086 AU (equivalent to approximately 100 µmol/min/L or 0.4 µmol/min/g). If the assay is done with a final substrate concentration of $1x10^{-3}$ M, the spontaneous hydrolysis is negligible.

8.5. Interpretation of Results

The interpretation of results is based on comparison of the individual value with the activity of the normal population for that species. The following risk levels have been indicated [2.3]:

Percentage inhibition of AChE:

0 - 20 % = zone of normal variation
20 - 50 % = presence of exposure or zone of reversible effects
50 - 100 % = life-threatening situation or zone of irreversible effects

Interpretation of plasma ChE results is reported to be more difficult than that of brain AChE because of high intra species variations. The correlation between plasma and brain ChEs should be considered only on a short-term basis since blood esterases recover quickly and brain ChEs remain depressed for a long period [2.3]. However, plasma ChE may be the best indicator for detection of low level of exposure to a known pesticidal application due to its more rapid and greater inhibition than brain AChE [2.2].

It is recommended that additional methods, such as ChE reactivation procedures or chemical analysis of residues in tissues or ingesta be used when no value for the normal population is available or when post-mortem handling conditions are unknown.

If, after the subtraction of spontaneous hydrolysis, ChE activity is smaller than the detection limit of the measurement technique (see Section 1), activities are reported as ND (not detected).

9. REPRESENTATIVE DOCUMENTS

Table of absorbance values and results obtained for typical analysis of control serum, brain homogenate and plasma are included in Sections 9.1, 9.2 and 9.3 respectively.

9.1. Typical Analysis of Control Serum

----- TIME-BASED SCAN TABLE -----

Date : 04-30-1996
Time : 10:54:59
Operator : My name

Sample Name : Precinorm E
Solvent Name :
Wavelengths : 406
Std Deviation : OFF

Run Time = 120 seconds
Cycle Time = 15 seconds
Start Time = 60 seconds
Intgr Time = 5.0 seconds

TIME/WVL	406
60.0	+0.10631
75.0	+0.13319
90.0	+0.15891
105.0	+0.18506
120.0	+0.21170

----- TIME-BASED SCAN TABLE -----

Date : 04-30-1996
Time : 10:54:59
Operator : My name

Sample Name : Precinorm E
Solvent Name :
Wavelengths : 406
Std Deviation : OFF

Run Time = 120 seconds
Cycle Time = 15 seconds
Start Time = 60 seconds
Intgr Time = 5.0 seconds

Zero Order Rate Calculation Results

Calculations from 0 to 120 seconds
Conversion Factor = 702000 Results Units = µmol/min/L

Rate = +1.7510E-03 AU/sec
(406) = +1.2292E+03 µmol/min/L
Relative SDEV = 0.2997 %
Y-Intercept = +0.0014 AU

9.2. Typical Analysis of Brain Sample (10 µL of bald eagle brain homogenate containing 250 mg of tissue/ mL)

----- TIME-BASED SCAN TABLE -----

Date : 04-29-1996
Time : 11:19:11
Operator : My name

Sample Name : 72356BA1
Solvent Name :
Wavelengths : 406
Std Deviation : OFF

Run Time = 120 seconds
Cycle Time = 15 seconds
Start Time = 60 seconds
Intgr Time = 5.0 seconds

TIME/WVL	406
60.0	+0.26898
75.0	+0.33258
90.0	+0.39716
105.0	+0.46097
120.0	+0.52429

----- TIME-BASED SCAN TABLE -----

Date : 04-29-1996
Time : 11:19:11
Operator : My name

Sample Name : 72356BA1
Solvent Name :
Wavelengths : 406
Std Deviation : OFF

Run Time = 120 seconds
Cycle Time = 15 seconds
Start Time = 60 seconds
Intgr Time = 5.0 seconds

Zero Order Rate Calculation Results

Calculations from 0 to 120 seconds
Conversion Factor = 5616 Results Units = µmol/min/g

Rate = +4.2601E-03 AU/sec
(406) = +2.3924E+1 µmol/min/g
Relative SDEV = 0.1344 %
Y-Intercept = +0.0134 AU

9.3. Typical Analysis of Plasma Sample (20 µL of bald eagle plasma)

----- TIME-BASED SCAN TABLE -----

Date : 04-09-1996
Time : 11:04:49
Operator : My name

Sample Name : 72356PA1
Solvent Name :
Wavelengths : 406
Std Deviation : OFF

Run Time = 120 seconds
Cycle Time = 15 seconds
Start Time = 60 seconds
Intgr Time = 5.0 seconds

TIME/WVL	406
60.0	+0.10426
75.0	+0.12437
90.0	+0.14456
105.0	+0.16475
120.0	+0.18437

----- TIME-BASED SCAN TABLE -----

Date : 04-09-1996
Time : 11:04:49
Operator : My name

Sample Name : 72356PA1
Solvent Name :
Wavelengths : 406
Std Deviation : OFF

Run Time = 120 seconds
Cycle Time = 15 seconds
Start Time = 60 seconds
Intgr Time = 5.0 seconds

Zero Order Rate Calculation Results

Calculations from 0 to 120 seconds
Conversion Factor = 702000 Results Units = µmol/min/L

Rate = +1.3373E-03 AU/sec
(406) = +9.3877E+02 µmol/min/L
Relative SDEV = 0.2366 %
Y-Intercept = +0.0241 AU

10. QUALITY ASSURANCE

10.1. Quality Control Sample

Precinorm E (Boehringer Mannheim) is a lyophilized protein-containing control serum to which enzymes have been added. It is intended for control of accuracy and precision in assay series and from day to day, for manual and automated analytical procedures. The ChE activity of a control serum is measured at the beginning, in the middle and at the end of a series of samples (i.e., 3 times a day). Control charts are prepared as described in the standard operating procedure SOP-BMK-DOC-03, which also details non-conforming criteria and corrective actions to be taken. Records of control samples are compiled on FORM-BMK-02a or b.

10.2. Accuracy

It is difficult to evaluate the accuracy of this method since there is no commercial reference material with the same matrix available, and also because very small differences in methodology will affect the results. However, when Precinorm E is analyzed with this method, the values are very close to the ones reported by the manufacturer (usually less than 10% difference).

To increase our confidence in the reliability of the value obtained, analyses are done in duplicate and repeated if results differ by more than 5% at normal levels and 15 % at low levels.

10.3. Sample Accountability

Possible sources of errors inherent in the collection, transport, and reception of specimen for analysis and any initial processing steps, such as separation of plasma, dissection, homogenization are recorded as described in SOP-BMK-DOC-04.

10.4. Linearity

Assays are done in the linear range of the spectrophotometer.

A 60 sec delay is allowed before recording the absorbance values to ensure linearity of the reaction.

Samples with very high activity ($\Delta A > 6.6 \times 10^{-3}$ Au/sec) may result in non-linear readings. In such cases the sample is diluted or 10 µL is used instead of 20 µL and the factor is corrected.

10.5. Reporting of Results

Results appearing on the worksheet form (FORM-BMK-13a or 13b) are compared with the original printout by the analyst and then by a second person. Data validation is insured by filling out FORM-BMK-32 "Data validation and reporting for ChE analysis - Checklist". Non-conforming situations are reported on form FORM-TP-06 and corrective actions are applied if necessary.

10.6. Randomization of Samples

Samples from the same project are not usually all analyzed the same day. To avoid any biased results, samples are analyzed at random, not taking into account the sample collection date or site.

10.7. Standard Operating Procedures (SOPs)

Other related SOPs used in this assay include:

- SOP-BMK-PROC-07 for pipet calibration
- SOP-BMK-PROC-01 for reagent solutions
- SOP-BMK-PROC-03 for glassware cleaning
- SOP-BMK-PROC-09 for temperature verification
- SOP-BMK-PROC-10 for balances calibration
- SOP-BMK-MAIN-01 for spectrophotometer calibration
- SOP-BMK-MAIN-03 for pH meter verification

Calibration protocols for pipets, thermometers, balances, pH meter and spectrophotometer are detailed in these procedures as well as the calibration frequency required. Results of the calibration and verification are then included in the Quality Assurance binder.

11. CRITICAL CONTROL POINTS

- Tissues should be kept on ice at all times and the duration of the assay should be kept to a minimum to prevent dissociation of carbamate-inhibited enzymes.

- The assay should be done in artificial room light, with elimination of daylight, because DTNB is light sensitive [2.9].

- Samples pertaining to a particular study must be processed and stored the same way.

- The catalytic activity of ChEs is temperature dependent so the experimental temperature has to be well monitored.

- Although this method is useful in diagnosing exposure to cholinesterase inhibiting substances such as organophosphorus and carbamate pesticides, it may yield results which underestimate the extent of ChE inhibition in tissues of animal exposed to carbamate pesticides, because the enzyme has the ability to reactivate spontaneously by decarbamylation. Precautions to minimize this reactivation of the enzyme must be taken otherwise results will not reflect *in vivo* ChE activity.

- ChE activity varies among species and the substrate concentration used in this method might not be appropriate. With the assay conditions described in this method, brain tissue should be analyzed with a final substrate concentration of $1x10^{-3}$ M to avoid inhibition of the enzyme by high concentrations of the substrate.

- Seemingly minor modifications of the Ellman method can influence the final ChE activity rates significantly and, consequently, comparison of ChE data between laboratories should be avoided unless methods are standardized [2.10] .

SPECTROPHOTOMETRIC DETERMINATION OF CHOLINESTERASE ACTIVITY IN WHOLE BLOOD

based on Augustinsson's method [2.1]

1. SCOPE AND FIELD OF APPLICATION

This method is a colorimetric method for measurement of cholinesterase (ChE) activity in avian whole blood. It is used in wildlife toxicology research to detect exposure to cholinesterase-inhibiting pesticides (organophosphorus and carbamates). A depression in ChE activity below the normal levels of this species is often used as evidence of exposure and, in the case of field mortalities, the cause of death [2.2].

2. REFERENCES

2.1. Augustinsson, K.B., Eriksson, H. and Faijersson, Y. (1978) A new approach to determining cholinesterase activities in samples of whole blood. *Clinica Chimica Acta*, 89, 239-252.

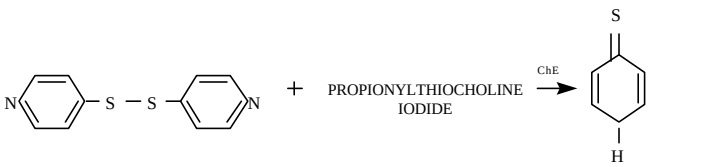
2.2. Ludke, J.L., Hill, E.W. and Dieter, M.P. (1975) Cholinesterase (ChE) response and related mortality among birds fed ChE inhibitors. *Arch. Environ. Contam. Toxicol.*, **3**, 1-21

2.3. Marden, B.T., Fairbrother, A. and Bennett, J.K. (1994) Interlaboratory comparison of cholinesterase assay measurements, *Environ. Toxic. and Chem.*, 13(11), 1761-1768.

3. PRINCIPLES AND DEFINITIONS

See Section 3.0 of method MET-BMK-CHE-01 for definitions of cholinesterase.

This method is based on the hydrolysis of propionylthiocholine and the spectrophotometric determination of the thiocholine produced by reaction with 4,4'dithiodipyridine (4-PDS). The reaction product 4-thiopyridone is measured at 324 nm, making the measurement in the presence of haemoglobin easier (the haemoglobin has an absorption maximum in the 400 nm region).



4,4'DITHIODIPYRIDINE

4'THIOPYRIDONE
(measured at 324 nm)

4. REAGENTS, SOLUTIONS, MATERIALS AND STANDARDS

SAFETY PRECAUTIONS

⇒ General safety rules and waste disposal procedures that apply to the Biomarker Laboratory must be followed (ref. Laboratory safety binder).

⇒ Adequate protective equipment must be used: lab coat, gloves, mask (when preparing solutions and tissues), cryogloves and face shield (when retrieving samples stored in liquid nitrogen).

⇒ Material Safety Data Sheets (MSDS) for the products used in the assay must be read.

4.1. Reagents

4.1.1. Sodium phosphate dibasic, Na_2HPO_4, M.W. 141.96, Fisher S-374

4.1.2. Potassium phosphate monobasic, KH_2PO_4, M.W. 136.09, Fisher P-285

4.1.3. 4,4' dithiodipyridine (4-PDS), M.W. 220.3, Sigma D-8136

4.1.4. Propionylthiocholine iodide (PrThChI), M.W. 303.2, Sigma P-2880

4.1.5. Methanol, HPLC grade

4.1.6. Purified water from the Milli RO / Milli-Q system

4.2. Solutions

4.2.1. *Sodium phosphate 0.05 M* - Dissolve 7.1 g Na_2HPO_4 in water and dilute to 1L.

4.2.2. *Potassium phosphate 0.05 M* - Dissolve 0.68 g KH_2PO_4 in water and dilute to 100 mL.

4.2.3. *Phosphate buffer 0.05 M, pH 8.0* (at room temperature) - Add 55 mL potassium phosphate solution to 945 mL of sodium phosphate solution. Adjust pH, if necessary, with H_3PO_4 or base.

4.2.4. *4-PDS, 10 mM in water* (stock solution) - Dissolve 22 mg 4-PDS in 1 mL of methanol, then complete volume to 10 mL with water. ***Shelf life: prepare fresh daily***

4.2.5. *4-PDS, 0.1 mM in phosphate buffer pH 8.0* (working solution) - Pipet 1 mL of the stock solution in a 100 mL volumetric flask and complete to volume with buffer.

4.2.6. *Propionylthiocholine iodide (PrThChI) 0.06 M* - Dissolve 184 mg PrThChI in water and dilute to 10 mL. ***Shelf life: prepare fresh daily***

4.3. Control Serum

See Section 4.3 of MET-BMK-CHE-01.

5. AUXILIARY EQUIPMENT

5.1. Glassware and Labware

5.1.1. Pipets (Biohit 5000 or Rainin and Gilson Pipetman 100 µL)

5.1.2. Glass fixed-needle Hamilton syringe, 500 µL

5.1.3. SMI pipet, 25 µL with glass capillaries

5.1.4. Disposable transfer pipets

5.1.5. Disposable acrylic cuvettes, 1 cm lightpath (Sarstedt 67.738)

5.1.6. Volumetric flasks, 100 and 500 mL, and 1 L

5.1.7. Graduated glass tube, 5 and 10 mL

5.1.8. Beakers, 250 mL and 1 L

5.1.9. Dispensing bottle (for purified water)

5.1.10. Ice bucket

5.2. Equipment

See Section 5.2 of method MET-BMK-CHE-01.

5.3. Instrumentation

See Section 5.3.1 to 5.3.3 of method MET-BMK-CHE-01.

6. SPECIMEN OR SAMPLE HANDLING REQUIREMENTS

It is advisable to use a freezer capable of maintaining -70°C (or lower) for storage. Long-term storage should be avoided in order to minimize problems associated with dehydration of the samples and stability of inhibited enzymes.

7. PROCEDURE

7.1. Sample Preparation

Hemolyze 25 µL of whole blood with 1.47 mL purified H_2O. Mix gently and keep cold.

- ***Minimum sample size recommended:*** 75 µL of whole blood.

7.2. Instrument Operating Parameters

- **Computer file path :** C:\HP8452
- **Mode :** F3 - Kinetic
- **Wavelength :** F1 - Single Wavelength (324,0)
- **Run Time :** F3 - RUN = 120 (readings from 60 sec to 120 sec with readings every 15 sec)
- **Stabilization Time:** F3 - START = 60 sec
- **Cycle and Integration Time :** F5 - CYCLE = 15 sec and INTGR = 5
- **Print format :** F2 - Tabular

7.3. Analysis

7.3.1. Turn on the spectrophotometer about 1 hour prior to the assay (period required to warm up the lamp).

7.3.2. Turn on the computer and enter the instrument operating parameters as described in Section 7.2.

7.3.3. Turn on the Lauda Circulator water bath, in order to maintain the cuvette temperature at 30°C, and the water bath used to pre-incubate the cuvettes.

7.3.4. Dispense 2.65 mL of 4-PDS/buffer solution (chromogen) in cuvette with Rainin or Biohit pipet and pre-incubate in water bath or directly in the spectrophotometer unit. Insert the temperature probe in the cuvette.

7.3.5. When the temperature has reached 27°C, add 300 µL of thoroughly mixed blood haemolysate using a 500 µL Hamilton syringe and gently mix the solutions with the probe.

7.3.6. When the temperature reaches 30.0 ± 0.2°C, remove the probe and press F8 - "SCAN BLANK" to zero the spectrophotometer at 324 nm. Then press F10 to exit the menu and F9 - "SCAN SCREEN".

7.3.7. Add 50 µL of substrate solution (propionylthiocholine iodide) with the 100 µL Pipetman pipet and start the scan by pressing F1 - "BEGIN SCAN".

7.3.8. Immediately mix the solutions using a disposable transfer pipet for about 15 sec and then allow sample to stabilize. The change in absorbance will be recorded for 1 min starting 60 sec after the addition of the substrate, as specified in Section 7.2.

7.3.9. Once the scan is completed, press F8 and enter the sample name.

7.3.10. Press F7 to access the calculation screen. Press F6 and enter the appropriate conversion factor (ref. Section 8.1) and F7 to specify the results units. Finally F1 to obtain the calculated results.

7.3.11. Print out the results by pressing F10 and F9. Then Press F10 to return to the screen “kinetic mode” to blank scan another sample.

7.3.12. Report the ChE activity measured on the worksheet (FORM-BMK-13e) and subtract the value of the spontaneous hydrolysis (ref. Section 8.2) and of the tissue blank (measured in the absence of the substrate) if applicable.

8. EXPRESSION OF RESULTS

8.1. Calculations

The rate of enzyme activity is reported in µmole of substrate hydrolyzed per minute per litre of whole blood, at 30°C. It is calculated by multiplying the change in absorbance per minute for the linear portion of the reaction by a factor of 1 818 000.

$$\text{Activity} = \frac{\Delta A/\text{min} \times \text{assay volume (mL)} \times 1\,000}{\text{abs. coeff.} \times \text{lightpath (cm)} \times \text{sample vol. (mL)}}$$

where: ΔA= change in absorbance
10^6 = to convert from M to µmole
60 = to convert sec in minute
abs. coeff. = absorbance coefficient for 4-thiopyridone at 324 nm.

for a total volume of 3.00 mL and a 1 cm lightpath the calculation is:

$$\frac{\Delta A/\text{min} \times .003\ \text{L} \times 10^6\ \mu\text{mol} \times 60\ \text{sec}}{\text{sec} \times 1.98\text{x}10^4\ \text{m}^{-1}\ \text{cm}^{-1} \times \text{M} \times 1\ \text{cm} \times \text{min}} = \Delta A/\text{min} \times 9.09 = \mu\text{mol/min/L}$$

To convert to 1 litre of whole blood, the value is multiplied by 2×10^5 because the amount of blood in the reaction mixture (3.00 mL) is 5 µL. Consequently, the factor to use is 1 818 000.

The activity value obtained is then corrected for the spontaneous hydrolysis of the substrate and the sample blank (ref. 8.2 and 8.3 below), which are calculated the same way.

8.2. Spontaneous Hydrolysis of the Substrate

Spontaneous hydrolysis of the substrate is determined at the beginning of each working day by adding 50 µL PrThChI directly to 2.95 mL of 4-PDS solution and measuring the change in absorbance in the absence of ChE enzyme. It is subtracted from the total hydrolysis value obtained. With the assay conditions of the present method, the ΔA/min is around 0.0086 AU (equivalent to approximately 100 µmole/min/L).

8.3. Interpretation of Results

The interpretation of results is based on comparison of the individual value with the activity of the normal population for that species.

In avian whole blood, the activity reported is due to the plasma enzymes and there is a greater intra-species variability then in the brain ChE. It is therefore recommended that additional methods, such as chemical analysis of residues in tissues or ingesta be used when no value for the normal population is available or when post-mortem handling conditions are unknown. Sequential sampling over a period of a few weeks can also be useful to detect recovery of the enzyme activity.

9. REPRESENTATIVE DOCUMENTS

Table of absorbance values and results obtained for typical analysis of whole blood haemolysate:

Note: 5.013 µL of blood in cuvette, consequently the factor is 1 813 000.

----- TIME-BASED SCAN TABLE -----

Date : 04-26-1996
Time : 13::31:16
Operator : My name

Sample Name : 72720HA1
Solvent Name :
Wavelengths : 324
Std Deviation : OFF

Run Time = 120 seconds
Cycle Time = 15 seconds
Start Time = 60 seconds
Intgr Time = 5.0 seconds

TIME/WVL	324
60.0	+0.05638
75.0	+0.06604
90.0	+0.07669
105.0	+0.08661
120.0	+0.09613

----- TIME-BASED SCAN TABLE -----

Date : 04-26-1996
Time : 13:31:16
Operator : My name

Sample Name : 72720HA1
Solvent Name :
Wavelengths : 324
Std Deviation : OFF

Run Time = 120 seconds
Cycle Time = 15 seconds
Start Time = 60 seconds
Intgr Time = 5.0 seconds

Zero Order Rate Calculation Results

Calculations from 0 to 120 seconds
Conversion Factor = 1813000 Results Units = µmol/min/L

Rate = +1.7510E-03 AU/sec
(324) = +1.2095E+03 µmol/min/L
Relative SDEV = 0.8068 %
Y-Intercept = +0.0163 AU

10. QUALITY ASSURANCE

10.1. Quality Control Sample

See Section 10.1 of method MET-BMK-CHE-01.

10.2. Accuracy

It is difficult to evaluate the accuracy of this method since there is no commercial reference material with the same matrix available. Precinorm E is used only to evaluate day to day analytical variability.

To increase our confidence in the reliability of the value obtained, analyses are done in duplicate and repeated if results differ by more than 5% at normal levels and 15 % at low levels.

In a lot of species there is non-enzymatic activity measured with whole blood tissue. This tissue blank is subtracted.

10.3. Sample Accountability

Possible sources of errors inherent in the collection, transport, and reception of specimen for analysis and any initial processing steps, are recorded as described in SOP-BMK-DOC-04.

10.4. Linearity

Assays are done in the linear range of the spectrophotometer. Spectrophotometer is verified regularly following SOP-BMK-MAIN-01.

A 60 sec delay is allowed before recording the absorbance values to ensure linearity of the reaction.

10.5. Reporting of Results

Results appearing on the worksheet (FORM-BMK-13e) are compared with the original printout by the analyst and then by a second person. Data validation is insured by filling out FORM-BMK-32 “Data validation and reporting for ChE analysis - Checklist”.

10.6. Randomization of Samples

Samples from the same project are not usually all analyzed the same day. To avoid any biased results, samples are analyzed at random, not taking into account the sample collection date or site.

10.7. Standard Operating Procedures (SOPs)

See Section 10.7 of method MET-BMK-CHE-01.

11. CRITICAL CONTROL POINTS

- Tissues should be kept on ice at all times and the duration of the assay should be kept to a minimum to prevent dissociation of carbamate-inhibited enzymes.
- Samples pertaining to a particular study must be processed and stored the same way.
- The catalytic activity of ChEs is temperature dependent so the experimental temperature has to be well monitored.
- Although this method is useful in diagnosing exposure to cholinesterase inhibiting substances such as OP and carbamate pesticides, it may yield results which underestimate the extent of ChE inhibition in tissues of animal exposed to carbamate pesticides, because the enzyme has the ability to reactivate spontaneously, by decarbamylation. Precautions to minimize this reactivation of the enzyme must be taken otherwise results will not reflect *in vivo* ChE activity.
- Background activity of samples may not be negligible in some species and should therefore be subtracted.
- Seemingly minor modifications of the method can influence the final ChE activity rates significantly and, consequently, comparison of ChE data between laboratories should be avoided unless methods are standardized [2.3].

SPONTANEOUS AND CHEMICAL REACTIVATION OF CHOLINESTERASE

based on the method of Martin *et.al.* [2.1]

1. SCOPE AND FIELD OF APPLICATION

This method is a biochemical test to differentiate between organophosphorus and carbamate inhibited brain and plasma cholinesterase. It is used in wildlife toxicology for the investigation of pesticide poisoning incidents and it is complementary to the chemical analysis of pesticide residues. In certain cases, even though pesticide exposure is the cause of the ChE inhibition, restoration of the enzyme is not always successful because some phosphorylated enzymes can dealkylate (age) and remain unaffected by nucleophilic reactivators such as 2-PAM. The amount of activity recovered depends on the nature of the exposure and on the assay parameters (e.g., incubation time, 2-PAM concentration).

2. REFERENCES

2.1. Martin, A.D., Norman, G., Stanley, P.I. and Westlake, G.E. (1981) Use of reactivation techniques for the differential diagnosis of organophosporus and carbamate pesticide poisoning in birds. *Bull. Environm. Contamin. Toxicol.*, 26, 775-780.

2.2. Pharmacia (1993), Instruction Sheet 52-1308-00, Edition AH.

3. PRINCIPLES AND DEFINITIONS

The inhibition of plasma and brain cholinesterase by organophosphorus and carbamate pesticides give rise to phosphorylated and carbamoylated enzyme intermediates. It has been established in many studies that carbamoylated enzymes are generally less stable and more susceptible to hydrolysis and tend to reactivate spontaneously. Conversely the phosphorylated enzyme, is more readily reactivated chemically by nucleophilic reagents such as 2-Pyridinealdoxime methiodide (2-PAM), due to the electrophilic nature of the phosphorous atom. In the case of carbamoylated enzyme, gel filtration separates the excess of pesticide from the enzyme intermediates and the excessive dilution of the sample encourage the spontaneous hydrolysis of the intermediate thus reactivating the enzyme. These differences are the basis of the procedure described in this document for distinguishing between organophosphorus and carbamate poisoning in birds.

4. REAGENTS, SOLUTIONS AND MATERIALS

SAFETY PRECAUTIONS

⇒ General safety rules and waste disposal procedures that apply to the Biomarker Laboratory must be followed (ref. Laboratory safety binder).

⇒ Adequate protective equipment must be used: lab coat, gloves, mask (when preparing solutions and tissues), cryogloves and face shield (when retrieving samples stored in liquid nitrogen).

⇒ Material Safety Data Sheets (MSDS) for the products used in the assay must be read.

4.1. Reagents

4.1.1. Sodium phosphate dibasic, Na_2HPO_4, M.W. 141.96, Fisher S-374

4.1.2. Potassium phosphate monobasic, KH_2PO_4, M.W. 136.09, Fisher P-285

4.1.3. 2-Pyridinealdoxime methiodide (2-PAM), M.W. 264.07, Aldrich P6,020-5

4.1.4. Purified water from the Milli RO / Milli-Q system

4.2. Solutions

4.2.1. *Potassium phosphate 0.05 M, pH 7.9* - see Section 4.2.3 of method MET-BMK-CHE-01.

4.2.2. *2-PAM 0.5 mM in water* - Dissolve 13.2 mg 2-PAM and dilute to 100 mL with water. ***Shelf life: prepare fresh daily***

5. AUXILIARY EQUIPMENT

5.1. Glassware and Labware

For reactivation with 2-PAM:

5.1.1. Pipet (Gilson Pipetman 200 µL or equivalent)

5.1.2. Test tubes, 12 x 75 mm, Fisher 14-961-26

5.1.3. Parafilm M ™

5.1.4. Volumetric flasks, 100 mL

For reactivation by gel filtration:

5.1.5. PD-10 Columns, Sephadex G-25 M, Pharmacia 17-0851-01 and support

5.1.6. Pipets (Biohit 5000 or equivalent and Gilson Pipetman 100 ΅L and 1000 µL or equivalent)

5.1.7. Plastic vials, 25 mL, Fisher 3-341-13A

5.1.8. Disposable transfer pipets

5.1.9. Graduated cylinder, 25 mL

5.2. Equipment

See Section 5.2 of method MET-BMK-CHE-01.

5.3. Instrumentation

See Section 5.3 of method MET-BMK-CHE-01.

6. HANDLING OF SPECIMENS

See Section 6.0 of method MET-BMK-CHE-01.

7. PROCEDURE

7.1. Chemical Reactivation with 2-PAM

7.1.1. For each sample two 12 x 75 mm disposable test tubes are required.

7.1.2. In tube 1, mix 100 ΅L of sample with 100 ΅L of 2-PAM solution 0.5 mM.

7.1.3. In tube 2, mix 100 ΅L of sample with 100 ΅L of purified water.

7.1.4. With each series of samples, prepare: a) one test tube containing 100 µL of water and 100 µL of 2-PAM and b) 2 test tubes containing a QC sample as described in 7.1.2 and 7.1.3.

7.1.5. Cover the test tubes with Parafilm M™ and incubate at ca 30°C one hour in water bath.

7.1.6. ChE activity of the restored enzymes is determined as described in Section 7.3.1 with 20 ΅L of the incubated mixture.

7.2. Spontaneous Reactivation by Gel Filtration

7.2.1. Column preparation

7.2.1.1. Remove the top cap and pour off excess liquid.

7.2.1.2. Cut off the bottom cap with scissors.

7.2.1.3. Support the column over a waste container and equilibrate the gel bed with ca 25 mL of the phosphate buffer pH 7.9.

7.2.2. Reactivation of cholinesterase

7.2.2.1. In a 12 x 75 mm test tube, dilute 100 ΅L of brain homogenate or plasma with 2.4 mL of phosphate buffer pH 7.9.

7.2.2.2. Add the diluted sample (in a total volume of 2.5 mL) to the column and discard the eluent.

7.2.2.3. Add 3.5 mL of phosphate buffer pH 7.9 to the column and collect the eluent containing the enzyme in a 25 mL plastic vial.

7.2.2.4. With a 25 mL graduated cylinder, dilute the eluent by adding 21.5 mL of phosphate buffer pH 7.9.

7.2.2.5. Cover, mix by inversion and store in a refrigerator at 4/C for at least 24 hours to allow the spontaneous reactivation to proceed.

7.3. Analysis

7.3.1. ChE activity determination after chemical reactivation with 2-PAM:

7.3.1.1. Analyze the samples as described in MET-BMK-CHE-01 (ref. Section 8.2.1 of the present method for conversion factors' details).

7.3.1.2. For samples containing 2-PAM, subtract the spontaneous hydrolysis obtained with tube containing 2-PAM and water (ref. Section 7.1.4 a).

7.3.1.3. Record results on form FORM-BMK-13c.

7.3.1.4. Analyze Precinorm E as described in MET-BMK-CHE-01 and for additional quality control, include a sample that is known to have been exposed to an organophosphorus compound with each series of samples.

7.3.2. ChE activity determination after spontaneous reactivation with gel filtration:

7.3.2.1. Analyze the samples as described in MET-BMK-CHE-01, with the following modifications:

a) Section 4.2.4: Prepare the DTNB as a 1.44 M solution by dissolving 57 mg in 100 mL phosphate buffer pH 7.9.

b) Section 7.3.4 and 7.3.5: Dispense 2.5 mL of the diluted brain or plasma sample (equivalent to 10 µL of the original plasma or brain homogenate) to the cuvette with the Rainin pipet. When the temperature has reached 27°C, add 520 µL of DTNB/buffer solution (chromogen) in cuvette with Pipetman.

d) Section 7.3.9: Ref. Section 8.2.2 of the present method for appropriate factor.

e) Section 8.4: Add 2.5 mL of phosphate buffer to cuvette. Bring to 27°C and add 520 µL of the DTNB/buffer solution.

7.3.2.2. For the control serum analysis, add 10 µL of Precinorm E to 2.5 mL of phosphate buffer, bring to 27°C and add 520 µL of the DTNB/buffer solution.

7.3.2.3. For additional quality control, include a sample that is known to have been exposed to a carbamate with each series of samples.

7.3.2.4. Record the results on form FORM-BMK-13d.

8. EXPRESSION OF RESULTS

8.1. Calculations

Refer to MET-BMK-CHE-01 for explanation of calculations.

8.2. Conversion Factors

8.2.1. Chemical reactivation with 2-PAM

Compared to its original concentration, the plasma or brain homogenate is diluted by a factor of 2. Consequently, using 20 µL of the diluted samples in the assay (see Section 7.3.1 of this document) is equivalent to 10 µL of the original sample and the conversion factors will then be:

- for plasma samples: 1 404 000
- for brain samples : 5 616

8.2.2. Reactivation by gel filtration

Compared to its original concentration, the plasma or brain homogenate is diluted by a factor of 2, consequently, using 2.5 mL of the diluted sample in the assay (see Section 7.3.2 of this document) is equivalent to 10 µL of the original sample and the conversion factors will be:

- for plasma samples: 1 404 000
- for brain samples : 5 616

8.3. Interpretation of Results

The interpretation of results is based on comparison of the ChE activity before and after reactivation. If the activity is higher after chemical reactivation with 2-PAM (tube 1) than the original value, this reactivation must be confirmed by determining the cholinesterase activity of the sample incubated with water (tube 2). If the enzyme reactivated in the presence of 2-PAM and did not reactivate after incubation with water, this is typical of an inhibition caused by an organophosphorus compound.

If reactivation occurred in the absence of 2-PAM the inhibitor is more likely to be a carbamate. This can be confirmed by reactivation with the gel filtration technique. If the pesticide is present in the sample in a relatively high concentration, the gel filtration technique will help the reactivation process in removing the excess of pesticide and diluting extensively the enzyme.

Note: If chemical reactivation with 2-PAM is not successful, it does not imply that there has been no exposure to organophosphorus pesticides - some

phosphorylated enzymes can spontaneously dealkylate (age) and in this form they remain unaffected by nucleophilic reactivators.

9. REPRESENTATIVE DOCUMENTS

N/A

10. QUALITY CONTROL

10.1. As described in Section 10 of MET-BMK-CHE-01, except that analyzes are not done in duplicate.

10.2. A control sample is analyzed with every series of samples reactivated. For the 2-PAM reactivation, it is a sample that has been exposed to an organophosphorus compound and for the gel filtration, it is a sample that has been exposed to a carbamate. These samples have reduced ChE activity following dosing experiments or were involved in pesticide intoxication incidents.

11. CRITICAL CONTROL POINTS

- As described in Section 11 of MET-BMK-CHE-01.
- The incubation of the sample with 2-PAM and with water must be done at the same time and following the same incubation conditions (time and temperature).

APPENDIX A

SOP-BMK-PROC-06

Brain Dissection for Cholinesterase Activity Determination

1. SCOPE AND FIELD OF APPLICATION

This procedure describes how to dissect brain which will then be used for cholinesterase (ChE) activity level determination. It is written specifically for avian species but the general procedure is the same for all species requiring brain ChE activity level determination.

2. PURPOSE

To insure that the dissection is done in a uniform manner by all analysts. If the dissection is not done properly, the results may be biased, because the enzyme is not distributed uniformly in the brain.

3. MATERIALS AND EQUIPMENT

3.1. Dissecting board

3.2. Scissors (angle dissecting scissors and/or surgical scissors)

3.3. Bone cutting shears and/or high-speed power tool (Dremel Multipro+5)

3.4. Homogenizing tubes (varying sizes, depending on the species)

3.5. Support and clamps (to secure head when power tool is used).

4. PROCEDURE

4.1. Using a pair of sturdy scissors, sever the head from the body at the skull vertebral column interface and pull the skin and feathers forward toward the beak. ***Caution****:* Mammals should be dissected and homogenized under fume-hood to avoid possibilities of disease transmission.

4.2. Using small pointed surgical scissors, insert the lower blade of the scissors through the interorbital septum and make a medial cut through the skull up the interorbital septum; snip across the narrow bridge of the interorbital septum (ref. **Figure 1** for diagram of skull and brain structures). Be careful to make small cuts with the scissors and direct the blade tips away from the brain to avoid damaging neural tissue. For raptor's brain with hard skull, a power tool with appropriate accessories can be used. If a power tool is used:

a) work under the fume-hood and use the protection shield;

b) insure that the bird's head is properly clamped on the support;

c) use both hands to hold the power tool;

d) avoid coming close to the bird's feathers with the power tool turned on;

e) orient the blades in the safest manner (away from you) and

f) consult owner's manual for detailed safety instructions.

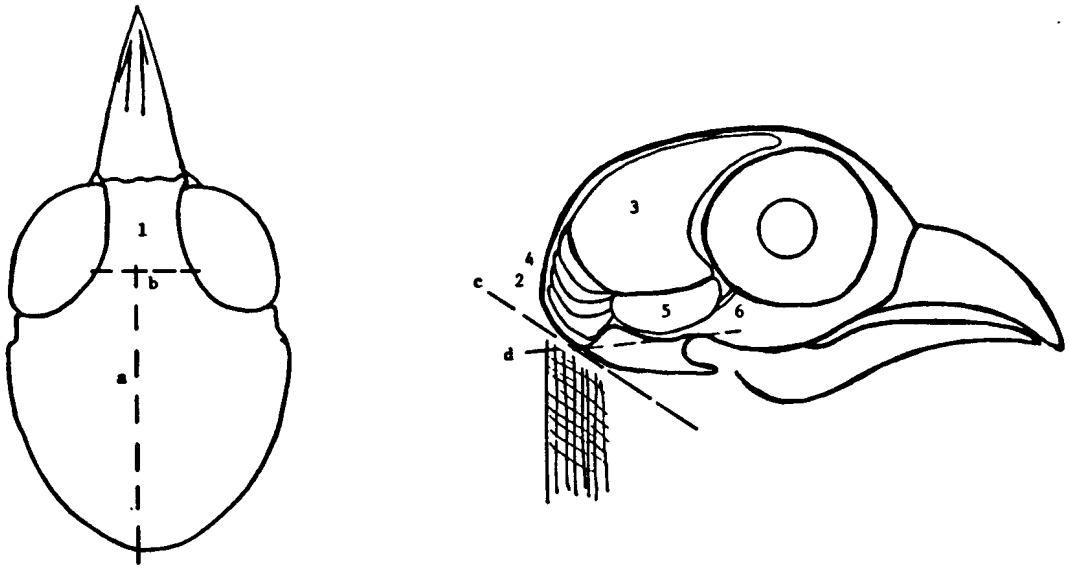
4.3. Insert the scissors through the foramen magnum and make a lateral cut through each exoccipital bone up to the ear opening.

4.4. The skull should now open as if on hinges; using the tip of a small spatula, gently free the brain from the surrounding connective tissue and sever the optic chiasma at the base of the skull with a slicing motion of the spatula. *Note:* The brain may be medially bisected to reduce size or for a split sample, however it should not be sectioned other than medially because of differences in brain area ChE activities.

4.5. Prepare brain tissue for analysis immediately after excision, as described in MET-BMK-CHE-01.

4.6. Thoroughly clean working table after manipulations with disinfectant solutions.

Figure 1. Diagrams of bobwhite skull and brain structures. - Dashed lines indicate cutting lines (from ASTM Subcommittee E47.04 on Wildlife Toxicology - Task Group E47.04.04 "Standard Practice for Measurement of ChE Activity in Animal Brain and Blood", Oct. 23, 1987



1. interorbital septum

2. foramen magnum

3. cerebral hemisphere

4. cerebellum

5. optic lobe

6. optic chiasma

a. cut from foramen magnum to interorbital septum

b. cut across interorbital septum

c. sever head below foramen magnum

d. cut through exoccipital bone to ear opening

APPENDIX B

FORMS

FORM-BMK-01 / August 1997

SAMPLE PREPARATION - Brain tissue for ChE assay

Analyst:	**Notebook no.:**	**Date:**
SOP no.:	**Page:**	

Project no.:	**Dissection: biomarker** ❑
Project leader:	**other** ❑

Homogenizing tube:	**70 ml**	❑	**Rotor no.:** **JA 18.1**	❑
	55 ml	❑	**JA 18**	❑
	15 ml	❑	**JA 21**	❑
	10 ml	❑		
	2 ml	❑	**Balance:** **top-loading**	❑
			analytical	❑

Lab. no.	Sample no.	Brain weight (g)	Vol. Triton/buffer (ml)	Remarks

FORM-BMK-13a / March 1999

CHE WORKSHEET - Final substrate [5x10-3 M]

Analyst:	Date:
Method:	

Project no.:	Notebook no.:	AThChI weight: ____ mg
Project leader:	Page:	completed to _____ ml

Spontaneous hydrolysis	factor: 702000	1) 2)	x=
	factor : 5616	1) 2)	x=

Precinorm E (QC) 1) 2) 3)	- hydr. spon. 1) 2) 3)	corrected for temperature (x .78) 1) 2) 3)	lot no. expected value:

LAB NO.	SAMPLE ID	USOX	REMARKS	CHE ACTIVITY	CORRECTED
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)

RESULTS VERIFIED
INITIALS .....

Storage location after assay:

FORM-BMK-13b / March 1999

CHE WORKSHEET - Final substrate [1x10-3 M]

Analyst:	Date:
Method:	

Project no.:	Notebook no.:	AThChI weight: ____ mg
Project leader:	Page:	completed to _____ ml

Precinorm E (QC)	lot no.
1) 2) 3)	expected value:

LAB NO.	SAMPLE ID	USOX	REMARKS	CHE ACTIVITY	CORRECTED
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)

RESULTS VERIFIED Storage location after assay:

INITIALS .....

FORM-BMK-13c / March 1999

CHE WORKSHEET - 2-PAM REACTIVATION

Analyst:	Date:
Method:	Final substrate concentration:

Project no.:	Notebook no.:	AThChI weight: ____ mg
Project leader:	Page:	completed to _____ ml

Spontaneous hydrolysis	factor:	1)	2)	x=
	factor (2-PAM+H2O):	1)	2)	x=
	factor :	1)	2)	x=
	factor (2-PAM+H2O):	1)	2)	x=

Precinorm E (QC)	- hydr. spon.	corrected for temperature (x .78)	lot no.
1) 2) 3)	1) 2) 3)	1) 2) 3)	expected value:

LAB NO.	SAMPLE ID	USOX	REMARKS	CHE ACTIVITY	CORRECTED
PAM				2-PAM	
				H2O	
PAM				2-PAM	
				H2O	
PAM				2-PAM	
				H2O	
PAM				2-PAM	
				H2O	
PAM				2-PAM	
				H2O	
PAM				2-PAM	
				H2O	
PAM				2-PAM	
				H2O	
PAM				2-PAM	
				H2O	
PAM				2-PAM	
				H2O	
PAM				2-PAM	
				H2O	

RESULTS VERIFIED Storage location after assay:

INITIALS .....

FORM-BMK-13d / March 1999

CHE WORKSHEET - GEL FILTRATION REACTIVATION

Analyst:	Date:
Method:	Final substrate concentration:

Project no.:	Notebook no.:	AThChl weight: mg
Project leader:	Page:	completed to _____ ml

Spontaneous hydrolysis	factor:	1)	2)	x=
	factor :	1)	2)	x=

Precinorm E (QC)	- hydr. spon.	corrected for temperature (x .78)	lot no.
1) 2) 3)	1) 2) 3)	1) 2) 3)	expected value:

LAB NO.	SAMPLE ID	USOX	REMARKS	CHE ACTIVITY	CORRECTED
GF					
GF					
GF					
GF					
GF					
GF					
GF					
GF					
GF					
GF					

RESULTS VERIFIED

INITIALS .....

Storage location after assay:

FORM-BMK-13e / March 1999

CHE WORKSHEET - Augustinsson's method

Analyst:	Date:
Method:	

Project no.:	Notebook no.:	PrThI weight: ____ mg
Project leader:	Page:	completed to _____ ml

Spontaneous hydrolysis	factor:	1)	x=
		2)	

Precinorm E (QC)	- hydr. spon.	lot no.
1) 2) 3)	1) 2) 3)	expected value:

LAB NO.	SAMPLE ID	USOX	REMARKS	CHE ACTIVITY	CORRECTED
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)
				1)	1)
				2)	2)

RESULTS VERIFIED Storage location after assay:

INITIALS .....

Over 50% recycled paper including 10% post-consumer fiber.