EPA Cholinesterase Methodologies Workshop

Abstracts

Sheraton Crystal City
Arlington, Virginia

December 4-5, 1991

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CHOLINESTERASE METHODOLOGIES WORKSHOP

Sheraton Crystal City
Arlington, Virginia
December 4-5, 1991

AGENDA

WEDNESDAY, DECEMBER 4, 1991

7:30AM-4:30PM	Participant and Observer Registration
8:30AM-8:40AM	Opening Remarks *Barry Wilson*
	Session I - Chemistry/Biology of Esterases **Session Chairperson - *David Lenz***
8:40AM-9:10AM	Characteristics of Esterases: Enzymology and Biology of Cholinesterases *Stephen Brimijoin*
9:10AM-9:20AM	Discussion
9:20AM-9:45AM	Factors Affecting Cholinesterase Inhibition *David Lenz*
9:45AM-10:00AM	Discussion
10:00AM-10:15AM	Break
	Session II- Methods for Measuring ChEI **Session Chairperson - *B. P. Doctor***
10:15AM-10:35AM	Overview/Microtiter Assay for Cholinesterase *B.P. Doctor*
10:35AM-10:550AM	Determination of Cholinesterase Activity in Plasma, Erythrocytes, and Brains of Experimental Animals Based on Ellman's Method *Paul Chin*
10:55AM-11:25AM	Discussion
11:25AM-11:45AM	Radiometric Method: Cholinesterase Measurements in Tissues from Carbamate-Treated Animals *Stephanie Padilla*
11:45AM-12:15PM	Discussion

Session III - Cholinesterase Measurements: Variabilities Observed in Results
Facilitator - *Barry Wilson*

2:00PM-2:45PM	Cholinesterase Data Collected from Bioassays Performed for Hazard Evaluations *Karen Hamernik*
2:45PM-2:55PM	Questions
2:55PM-3:40PM	Cholinesterase Data Obtained from Historical Control Animals *Jeffrey Charles*
3:40PM-4:00PM	Break
4:00PM-5:00PM	Discussion
5:00PM	Wrap-Up *Barry Wilson*

THURSDAY, DECEMBER 5, 1991

7:30AM-8:30AM	Observer Registration
	Recapitulation of Day I
8:30AM-8:40AM	Chemistry/Biology Session *David Lenz*
8:40AM-8:50AM	Analytical Methods *B.P. Doctor*
8:50AM-9:00AM	Historical Control and Toxicity Testing Bioassay Data *Barry Wilson*

Session IV - Protocol/Guidelines for ChE Determinations: Future Research Needs
Session Chairperson - *Stephanie Padilla*

9:00AM-9:10AM	Introduction *Stephanie Padilla* (not confirmed)
9:10AM-9:30AM	Cholinesterase Assay Strawman Protocol *Brian Dementi*
9:30AM-10:00AM	Discussion
10:00AM-10:15AM	New Developments in Laboratory Testing *Juergen Thyssen*
10:15AM-10:30AM	Existing Guidelines for Assessing Cholinesterase Inhibition Caused By OPs and Carbamates *Bruce Jaeger*
10:30AM-10:45AM	Discussion
10:45AM-11:00AM	Break

Session V - Future Directions
Session Chairperson - *Hugh Tilson*

11:00AM-11:20AM	Cholinesterase Inhibitors: Ongoing and Proposed Research Needs *Hugh Tilson*
11:20-11:40AM	New Opportunities in Cholinesterase Testing Paradigm *Dan Hanke*
11:40AM-12:00PM	Discussion
12:00PM-12:20PM	Summary of Workshop *Barry Wilson*
12:20PM-1:30PM	Formal Workshop Adjourns/Lunch
1:30PM-4:00PM	Executive Session (Drafting of Summary Reports)

CHARACTERISTICS OF ESTERASES: ENZYMOLOGY AND BIOLOGY OF CHOLINESTERASES

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Among the many classes of esterases, the most important from a physiologic and toxicologic standpoint are the B-esterases, acetyl-cholinesterase (AChE) and butyrylcholinesterase (BuChE). AChE, concentrated in synapses, is necessary for cholinergic neurotransmission, but there is a margin of safety and a significant fraction of the AChE activity must be inhibited before signs of toxicity are evident. There is no known function for AChE in non-synaptic sites like the erythrocyte or for BuChE in plasma and elsewhere, but the activities of these enzymes can be used as indicators of exposure to environmental anticholinesterases. To predict and interpret the effects of anticholinesterases on AChE and BuChE, understanding of the tissue distribution of the enzymes and their molecular forms is required. Tissue distribution has major implications for the accessibility of enzyme to toxicants, especially in view of blood-brain and blood-nerve barriers that block the free diffusion of molecules with low lipid-solubility. The occurrence of multiple molecular forms of AChE and BuChE is also of crucial importance. These molecular forms are best understood as assemblies of identical, or nearly identical, catalytic subunits, with or without accessory molecules that promote attachment to the cell surface membrane or the extracellular synaptic basal lamina. The functional significance and regulation of the molecular forms of AChE and BuChE are still being elucidated, but it is already clear that the enzyme forms have characteristic cellular locations and rates of renewal. These factors affect both the extent of inhibition and the rate of recovery after anticholinesterase exposure. Such considerations underscore the need for appropriately designed tests to determine (a) potential anticholinesterase activity of target compounds in vivo; (b) biological risks associated with environmental use of these compounds; and (c) actual adverse effects in individuals and populations at risk. Conventional activity-based assays of AChE and BuChE are suitable tests for most purposes, while more elaborate immunoassays can be used in order to define the levels of cholinesterase protein.

FACTORS AFFECTING CHOLINESTERASE INHIBITION

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Cholinesterases (ChEs) have been classified by their preference to catalyze a particular substrate. Acetylcholinesterase (AChE), which is found in red cells, the central nervous system, and in nerve endings, displays a marked preference for the neurotransmitter acetylcholine. Butyrylcholinesterase (BuChE), which is most prevalent in the plasma as well as a variety of tissues such as heart, exhibits less substrate specificity but has maximal catalytic activity for the hydrolysis of butyrylcholine. Both enzymes have been described as having an esteratic site and an anionic site. With the recent elucidation by Sussman and coworkers of the 3-dimensional structure of torpedo AChE the traditional model of the active site has been revised. While an esteratic site was confirmed, the anionic site was found to be comprised of aromatic amino acids rather than any amino acid with a formal positive charge. This finding may require some reexamination of the large body of inhibition data that exists for AChE with respect to structure/activity relationships. However, at this time it does not seem likely that the classical inhibition kinetic model based on Michaelis-Menton kinetics will have to be abandoned.

ChEs are inhibited by compounds which can phosphorylate or carbamylate the active site. Many of the studies were carried out in vitro. Earlier studies that were carried out in vivo were often incomplete with respect to a detailed analysis of the pharmacokinetics of the inhibition of ChE in a particular tissue. Within the last few years however there have been a series of pharmacokinetic studies of the inhibition of ChE (usually AChE) which have examined the effect of the route of administration of the inhibitor, e.g., intramuscular, inhalation, or intravenous, in several animal species. The effect of naturally occurring target enzymes other than ChE, such as carboxylesterase (CaE) on the pharmacokinetics of AChE inhibition has been determined as have the pharmacokinetics of diastereomeric ChE inhibitors. In all studies, the detoxication of organophosphorus ChE inhibitors has been markedly affected by the relative tissue concentrations of CaE. It was also determined that ChE inhibitors can affect blood flow to different organ systems and that this correlated with the relative extent of ChE inhibition in these organ systems.

Often the extent of ChE inhibition has been determined by measuring ChE activity in a blood sample, either whole blood or plasma, and then those results extrapolated to the whole organism. Recent studies by Shih, Jimmerson, and Maxwell have revealed that there is a poor correlation between blood AChE activity and plasma BuChE activity with respect to each other or to central nervous system (whole brain) AChE activity. These initial findings were confirmed in subsequent studies and expanded upon to reveal a poor correlation between blood AChE or BuChE and regional brain AChE activity, suggesting that measurement of blood ChE activity after exposure to a ChE inhibitor is a poor indicator of central toxicity. These investigators did report a good correlation between brain AChE activity and toxic signs. They further noted that while blood ChE activity did not correlate well with brain regional AChE the former was the initial site of ChE inhibition and as such provided a very sensitive indicator of exposure to a ChE inhibitor.

Studies of ChE inhibition and hence the toxicity of various ChE inhibitors in vivo have

revealed a large variability between animal models. This has often been attributed to 'species variation' with little additional explanation. Initial studies by Fonnum and Sterri identified a role for CaE in affecting inhibitor toxicokinetics. This was expanded upon by Maxwell who demonstrated that the concentration of CaE in circulation could explain the difference in toxicity of ChE inhibitors in different species. The enzyme CaE is particularly prevalent in rodent blood but almost absent in human and non-human primate blood. By selectively inhibiting the activity of CaE, the differences in species toxicity could be eliminated. These data must be interpreted carefully however, because too high of dose of the CaE inhibitor can also affect ChE activity.

Despite the numerous variables that affect the inhibition of ChE, e.g., form of ChE affected, route of administration of inhibitor, concentration of endogenous CaE, alterations in blood flow, stereochemistry of the inhibitor, correlation between blood ChE activity and toxicity, inhibitor pharmacokinetics, and animal species, there have been attempts to define some unifying models whereby inhibitor concentration and extent of ChE inhibition in a particular tissue can be determined. Most of these efforts have centered around the use of physiological pharmacokinetic modeling. The difficulty with this approach is that much of the data needed to solve the large set of simultaneous differential equations that describe the physiologic process are not available. However in one of the few cases where the data were available, Maxwell and coworkers were able to use this approach to correlate inhibitor concentration with the extent of ChE inhibition in a variety of tissues. Their preliminary success suggests that this approach may offer a method of extrapolating toxicity data across species up to and including humans.

References

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MICROTITER ASSAY FOR CHOLINESTERASES

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A microtiter plate adaptation of the Ellman colorimetric procedure for measurement of acetyl- or butyrylcholinesterase activity is described (Doctor et al. (1987) Anal. Biochem., 166:399-403). This method uses ELISA plate reader for rapid analysis of multiple samples and is particularly suitable for analysis of cholinesterase activity on sucrose gradients and column chromatography fractions. This procedure is rapid, sensitive, and does not require radioactive material.

This procedure has been used for determination of ChEs in whole blood and plasma obtained from mice, rats, monkeys, and humans. It is also used in the simultaneous determination of protein and AChE of the same sample and to determine the inhibition of ChE activity by monoclonal antibodies. It is further modified to measure trace amounts of organophosphates.

DETERMINATION OF CHOLINESTERASE ACTIVITY IN PLASMA, ERYTHROCYTES, AND BRAINS OF EXPERIMENTAL ANIMALS BASED ON ELLMAN'S METHOD

Byong Han (Paul) Chin

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The method described by Ellman and coworkers (1961) is the principal method used in U.S. laboratories for determining plasma, RBC, and/or brain cholinesterase (ChE) activity in both human and laboratory animals. Among the numerous methods, this method has been widely used because this method is easily automated and the automated method is reproducible, precise, and more easily performed than the manual method. The method uses acetylthiocholine iodide as substrate which is hydrolyzed to thiocholine iodide and acetic acid by the cholinesterase activity in biological tissues. The liberated thiocholine reduces color reagent, 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) to 5-thio-2-nitrobenzoic acid which is measured spectrophotometrically. This presentation will cover the following subjects related to the Ellman's method: basis of reactions; optimal concentrations for substrate, DTNB, and buffer; optimal pH; and spectrophotometric measurement of the end product. In addition, two most commonly used automated procedures, which are based on a continuous flow system (e.g., Technicon AutoAnalyzer) and centrifugal system (e.g., CentrifiChem), will be reviewed in terms of their instrumentation (e.g., reaction time, reaction temperature, and an absorbance reading); sample output (no. of samples analyzed/hour); and reproducibility of analysis.

CHOLINESTERASE MEASUREMENTS IN TISSUES FROM CARBAMATE-TREATED ANIMALS

Stephanie Padilla

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Between 1964 and 1975, many different investigators, using diverse approaches, attempted to develop a sensitive, quick, and safe assay for cholinesterase activity using radiolabeled substrate. The culmination of these efforts was a publication in 1975 by Johnson and Russell describing the radiometric method which is still used today. Briefly, the entire assay takes place in a scintillation vial with the tissue and substrate ([^{3}H]acetylcholine) combined in a total volume of 100 μL at the bottom of the vial, acidic buffer is added to stop the reaction, and the scintillant (approximately 5 ml) is then added to extract the radiolabeled product. When placed in a scintillation counter, only the substrate that has been hydrolyzed is counted.

A spectrophotometric assay, by its nature, requires extensive dilution of the tissue in order for proper passage of the beam of light; therefore, because of this necessary dilution step, the spectrophotometric method may underestimate the amount of cholinesterase inhibition in tissues from carbamate-treated animals. On the other hand, because the radiometric method may be conducted on undiluted or minimally diluted tissues, it would superior for determining cholinesterase activity in tissues from carbamate-treated animals. In our laboratory we routinely use both type of assays to measure cholinesterase activity in tissues collected from animals: the spectrophotometric assay (i.e., Ellman assay [Ellman et al., Biochem. Pharm., 7:88-95]); and a radiometric assay (Johnson and Russell, Anal. Biochem., 64:229-239, 1975). Using these two assays to determine cholinesterase activity in the same tissue samples, we have determined that the Ellman method consistently underestimates cholinesterase inhibition in tissues from carbamate-treated animals. We have, however, as have other laboratories, developed strategies for conducting Ellman assays on carbamate-treated tissues and will make recommendations for storage, preparation, and analysis of tissues from carbamate-treated animals.

ABSTRACT

CHOLINESTERASE DATA COLLECTED FROM BIOASSAYS PERFORMED FOR HAZARD EVALUATIONS

Karen L. Hamernik, Ph.D.
HED Cholinesterase Workgroup Representative
Office of Pesticide Programs
United States Environmental Protection Agency

Members of the Health Effects Division (HED) Cholinesterase Workgroup have prepared a document entitled "Measurement of Cholinesterase Activity in Toxicological Studies Submitted to the Environmental Protection Agency in Support of Pesticide Registration: Analysis of Data Variability. A Summary Report." The document represents the results of a first effort by the Workgroup to examine the reliability of the methods being used to measure cholinesterase activity in studies submitted to HED/OPP as part of the regulatory process. Major objectives were to identify the methods, protocols, standard operating procedures, and study designs used by laboratories performing plasma, erythrocyte, and brain cholinesterase bioassays for submitted studies, to analyze the variability in the data, and to attempt to identify factors contributing to the variability observed.

Twenty-eight (28) studies from HED toxicology files, 16 rat and 12 dog, were finally chosen for analysis after certain selection criteria had been applied. Test materials were organophosphorus or carbamate pesticides. Most of the studies were 1 year (dog) and 2 year (rat) feeding studies although some shorter term studies were included. In all studies, the Ellman method or a modification thereof, had been used to measure cholinesterase activity. The availability of a Standard Operating Procedure (SOP) from the testing laboratory was an important, although not absolute, factor in determining which studies were analyzed.

An overview of the modifications in the Ellman method used in the studies was prepared from information contained in the SOPs and hardcopies of the study reports.

Summarization and comparison of the cholinesterase assay data and data variability within and among studies was facilitated by the use of statistical measures such as the mean and standard deviation, coefficient of variation, median, and range.

Highlights of the modification overview and data evalutions will be presented during the talk.

CHOLINESTERASE DATA OBTAINED FROM HISTORICAL CONTROL ANIMALS

Jeffrey M. Charles

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Working with EPA, the National Agricultural Chemicals Association (NACA) provided historical control data on plasma, red blood cell (RBC), and brain cholinesterase (ChE) from six laboratories chosen by EPA. The laboratories included: Bush Run Research Center, Export, Pennsylvania; CIBA-Geigy, Summit, New Jersey; Haskell Laboratories, Newark, Delaware; Hazleton Washington, Vienna, Virginia; Hazleton Wisconsin, Madison, Wisconsin; and Mobay Corporation, Stilwell, Kansas. The laboratories provided the data from all rat and dog studies (that included cholinesterase determinations) initiated since January 1, 1986. The data provided included all of the individual animal data obtained at all time points in the study. Written methodologies, Standard Operating Procedures (SOPs), and/or protocols documenting the clinical methodologies employed in performing the ChE assays on plasma, RBC, and brain were also provided. All modifications of the procedures used over this 5-year period were provided.

A total of 78 studies were provided by the NACA member laboratories. Of the 56 rat studies provided, 28 were subchronic studies of 2 to 18 weeks in duration, 16 were reproduction studies of 9 to 22 months of duration, and 12 were 2-year chronic studies. Data from 22 dog studies were provided. Of these, 10 were subchronic studies of 2 to 13 weeks in duration and 12 were 1-year chronic studies.

In all the studies examined, ChE activity was assayed according to the modified method of Ellman et al. Parameters used to evaluate ChE variation included mean $\pm$ S.D. and the coefficient of variation (CV). CV is a statistical index that has been used in the analysis of non-uniform parameters such as plasma ChE in animal studies.

Data will be presented describing the variability in the methods used in the six laboratories in determining plasma, RBC, and brain cholinesterase activity in rats and dogs. Comparison will be made within each laboratory and within given studies.

ABSTRACT

CHOLINESTERASE ASSAY STRAWMAN PROTOCOL
Brian Dementi, Ph.D.
U.S.E.P.A.

A presentation based upon the Eliman, et al. cholinesterase methodology, which examines the various parameters in the assay in light of contemporary literature and industrial procedures with the objective of providing guidance in drafting a more definitive assay procedure for routine cholinesterase assays. This involves an examination of issues pertaining to many of the parameters in the assay itself, as well as to those having to do with the obtaining, preparation and storage of tissue samples for assay of cholinesterase activity.

Examples of topics under discussion include acetylthiocholine substrate concentration, non-enzymatic hydrolysis of substrate, dithionitrobenzoate indicator and its stability under various conditions, alternative indicators, spectrophotometer wavelength selection, hemoglobin absorption interference, assay temperature, enzyme unitology, factors of concern in assaying blood cholinesterases including in particular the handling of erythrocytes, use of anticoagulants, non-ionic detergent, distinguishing between butyrylcholinesterase and acetylcholinesterase in blood and other tissues such as brain, etc.

EXISTING GUIDELINES FOR ASSESSING CHOLINESTERASE INHIBITION CAUSED BY OPS AND CARBAMATES

Bruce Jaeger

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Guidance for conducting cholinesterase assays in laboratory rodents and dogs was outlined in 1959 by FDA in their booklet: "Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics (edited 1975)". In 1972, EPA outlined, for the first time, its own proposed guidelines for conducting cholinesterase assays. These were expanded in 1978 to make cholinesterase measurements a requirement for registration of OPs and carbamates. In 1982 and 1984, EPA revised its guidelines but provided very little guidance with respect to conducting cholinesterase assays. Outlines of these guidance documents are presented. None of them provide reference to assay procedures, i.e., Michael, Ellman, etc., or for tissue preparation or timing of cholinesterase determination for carbamates. Much of this latter information has been developed over the years on a case by case basis for each OP or carbamate pesticide. Guidance for development and expansion of cholinesterase bioassay procedures is solicited.

CHOLINESTERASE INHIBITORS: ONGOING AND PROPOSED RESEARCH NEEDS

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Cholinesterase inhibitors are used in many countries to control a variety of pests. These agents are designed to produce neurotoxicity in the target organism and humans may manifest neurotoxic signs and symptoms following accidental exposure. During the last four years, various workshops, colloquia, and briefings have been sponsored by EPA to address scientific issues concerning regulation of these chemicals. During the course of these meetings, several research needs were identified.

One of the most difficult questions concerns the definition of an adverse effect. Although there is some consensus that inhibition of brain cholinesterase may be adverse, more research is needed to determine the neurotoxicity associated with inhibition of blood cholinesterase. There is consensus that inhibition of blood cholinesterase is a valid indicator of exposure. In addition, research is needed to determine the relationship between blood and brain cholinesterase levels, the role of various esterases, and possible regional differences in sensitivity to the cholinesterase inhibitors. Other endpoints of neurotoxicity, particularly cognitive dysfunction, need to be developed and validated. A number of studies have indicated that developing organisms may be differentially sensitive to the effects of cholinesterase inhibition. In addition, there is some evidence that repeated exposure to cholinesterase inhibitors may accelerate the aging process. Other research is addressing why tolerance develops to some, but not all, effects following repeated exposure and if residual neurotoxicity exists following cessation of exposure. Research also is needed to determine the relationship between noncholinergic effects of these agents with the manifestation of neurotoxicity. Recent research has indicated that in vitro methodologies might have utility in the screening of cholinesterase inhibitors for neurotoxicity, as well as in investigations of structure-activity relationships and chemicals mixtures.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF PESTICIDE PROGRAMS
HEALTH EFFECTS DIVISION

CHOLINESTERASE METHODOLOGIES WORKSHOP DECEMBER 3-5 1991
SHERATON CRYSTAL CITY HOTEL, ARLINGTON, VIRGINIA

NEW OPPORTUNITIES IN CHOLINESTERASE TESTING PARADIGMS

DAN WOLF HANKE, PH. D.

ABSTRACT

With advancing technologies come new research opportunities for testing and development within the general field of the cholinergic synapse. Among those opportunities are the use of cloned and purified cholinesterases (ChEs) as well as cloned nicotinic and muscarinic receptors and a synthetic receptor. There are also opportunities that lie within the purview of intra-neuronal factors affecting developmental aggregation of acetylcholine receptors (AChRs) and exocytosis of acetylcholine (ACh) at nerve terminals attendant to nerve impulse transmission. My presentation today, however, focuses on the development of three ChEs and their possible use in an in vitro screening paradigm with all the attendant advantages and disadvantages associated with in vitro approaches. Two of the ChEs are human recombinant acetylcholinesterase (AChE) and human recombinant butyrylcholinesterase (BChE) cloned from brain and liver cDNA libraries respectively by Mona Soreq et al. at the Hebrew Un of Jerusalem in Jerusalem, Israel. The third ChE is AChE purified from fetal bovine serum via affinity chromatography on a procainamide-sepharose matrix by BP Doctor et al. at the Walter Reed Army Institute of Research (WRAIR) in Washington, DC. The use of these ChEs in an in vitro testing paradigm could rapidly and inexpensively generate reproducible, comparative toxicity data on pesticides. The pesticide inhibition data would be evaluated relative to AChE or BChE as well as relative to standard reference organophosphorus (OP) and carbamate compounds. Determination of the kinetic constants for ChE inhibition could provide information on acute toxicity. The availability of pure human and bovine ChEs may assist in directing end-use pesticide products towards appropriate applications peculiar to human or animal exposures respectively. The use of these three ChEs in an in vitro pesticide testing paradigm could help to standardize laboratory procedures, to generate reliable historical reference data within and across laboratories, and to assist in prioritizing pesticides for more cost-effective running the gauntlet of requisite animal studies.

CHOLINESTERASE METHODOLOGIES WORKSHOP

Sheraton Crystal City
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PRESENTERS' OUTLINES

Cholinesterase Methodologies Workshop
December 3-5, 1991

EPA Workshop on Cholinesterase

CHARACTERISTICS OF ESTERASES - ENZYMOLOGY AND BIOLOGY OF CHOLINESTERASES by W. S. Brimijoin, Ph.D.
Professor of Pharmacology
Mayo Clinic
Rochester, MN

I. INTRODUCTION
- A. Overview of Esterases
  - 1. Enzymology of Esterase Classes
    - a) A-Esterases (Arylesterase)
    - b) B-Esterases (AChE, BuChE, Carboxylesterase)
    - c) C-Esterases (Acetylesterase)

II. GENERAL CONSIDERATION OF CHOLINESTERASE
- A. Molecular Forms
  - 1. Structural Organization
  - 2. Dynamic Interrelations
  - 3. Molecular Biology
- B. Cellular and Subcellular Distribution
  - 1. Intracellular Sites
  - 2. Membrane Anchoring
  - 3. Extracellular Sites
- C. Functional Roles
  - 1. Synaptic
  - 2. Other
- D. Brief Introduction to Assay Methods

III. ACUTE AND CHRONIC INHIBITION
- A. Accessibility to Inhibitors
  - 1. Routes of Inhibitor Access
  - 2. Blood vs. Peripheral Tissues
  - 3. Central Nervous System
- B. Turnover and Replacement
- C. Signs of Inhibition
  - 1. Overt Signs
  - 2. Biochemical Determinations on Tissues and Fluids
  - 3. Combined Assays for Enzyme Activity and Immunoreactivity

IV. CONCLUSION

EPA Workshop on Cholinesterase Methodology

Session I

FACTORS AFFECTING ChEI by David E. Lenz

I. INTRODUCTION
- A. Three-Dimensional Structure
  - 1. Active Site
  - 2. Channel
- B. Classic Kinetics of Inhibition
- C. Issues of Importance
  - 1. Metabolism/Pharmacokinetics
  - 2. Blood ChE Activity vs CNS
  - 3. CaE Activity vs Species Toxcity

II. METABOLISM/PHARMACOKINETICS
- A. Classic Studies of Where Organophosphorus (O) Compounds Bind
- B. Recent OP/AChE Pharmacokinetic Studies
  - 1. Billy Martin
  - 2. David Lenz
    - a) CaE Inhibitor Absent
    - b) CaE Inhibitor Present
  - 3. Hendrick Benschop
    - a) Stereoisomer Considerations
    - b) Species Differences
- C. Blood Flow and Affect on ChE Inhibition in Tissue

III. ACTIVITY OF ChE IN BLOOD vs CNS
- A. Source of Enzyme vs Activity After Inhibition
  - 1. Blood AChE/BuChE
  - 2. Brain ChE
- B. Activity vs Behavioral Effects
  - 1. Blood ChE
  - 2. Regional Brain ChE
  - 3. Behavioral Measurements

IV. CaE ACTIVITY vs TOXICITY
- A. Effect of Dose of Inhibitor on CaE vs ChE Activity
- B. Effect of CaE Activity on Species Toxicity

V. SUMMARY
- A. Recapitulation of Problem in Light of Current Information
- B. The Potential of Physiological Pharmacokinetic Modeling to Extrapolate from Extent of ChE Inhibition to Toxicity

METHODS FOR MEASURING CHOLINESTERASE INHIBITION AN OVERVIEW

B. P. Doctor, Director, Division of Biochemistry
Walter Reed Army Institute of Research

I. INTRODUCTION

A. Methods: Ellman, Titermetric, Radiometric, (Johnson and Russell), Others

1. Principles of each method
2. Areas of applicability for each method
3. Cost, Ease, reliability, and reproducibility
4. Data collection, interpretation and time factor

B. Factors: (Effecting Ellman Method)

1. Temperature of reaction
2. Reactants, substrates
3. Reaction volume, buffer, pH
4. Sensitivity to light
5. Wavelength
6. Interference by presence of contaminants
7. Enzyme standard
8. Hydrolysis (non-enzymematic) of substrates

C. Sample Preparation

1. Blood, plasma, erythrocytes
2. Dilution, how much in what media
3. Haemoglobin interference
4. Acquisition, transportation, storage, shelf-life
5. Periodicity (more than one sample)
6. Brain, muscle other

D. Additional Considerations

1. Rate constants of carbamates
2. Rate constants of organophosphates
3. Use of inhibitors to distinguish various ChEs
4. Distribution, clearance and Circulation rate of carbamates and OP's
5. One enzyme source for standard

E. Alternate Methodology.

1. Modified Ellman method/Micro-titer plate assay
2. Biosensor techniques
3. Other

CHE WORKSHOP (Dec. 3-5, 1991)

OUTLINE

DETERMINATION OF CHOLINESTERASE ACTIVITY IN PLASMA, ERYTHROCYTES, AND BRAINS OF EXPERIMENTAL ANIMALS BASED ON ELLMAN'S METHOD

Byong Han (Paul) Chin, Health Effects Division, Office of Pesticide Programs
U.S. Environmental Protection Agency, Washington, DC 20460

I. INTRODUCTION

- A. Commonly used methods for determining cholinesterase activity in both human and laboratory animals
  1. Spectrophotometric method of Ellman and co-workers (1961)
  2. Electrometric method of Michel (1949)
- B. Other methods
  1. Titrimetric method
  2. Radiometric method
  3. Fluorimetric method

II. ELLMAN'S METHOD

- A. Basis of reactions
- B. Conditions of assay
  1. pH
  2. Color reagent, 5,5-dithio-bis-2-nitrobenzoic acid (DTNB)
  3. Substrate concentration
  4. Buffer concentration

III. INSTRUMENTAL ANALYSIS BASED ON ELLMAN'S METHOD

- A. Based on continuous flow system (e.g., Technicon AutoAnalyzer)
- B. Based on centrifugal system (e.g., CentrifiChem)
  1. Instrumental settings (e.g., reaction time, reaction temperature, and an absorbance reading)
  2. Sample output (No. of samples analyzed/hour)
  3. Reproducibility of analysis

Use of the Radiometric Method for Cholinesterase Measurements: Advantages and Disadvantages

by

Stephanie Padilla, Ph.D.
Neurotoxicology Division
U.S. Environmental Protection Agency
Research Triangle Park, NC 27711

I. BRIEF HISTORY OF THE DEVELOPMENT OF THE METHOD

II. OUTLINE OF THE PRESENT METHOD

III. DISADVANTAGES OF THE RADIOMETRIC METHOD

A. Endpoint Reaction
B. Employs a radioactive isotope
C. Requires a scintillation counter
D. Generates hazardous waste

IV. ADVANTAGES OF THE RADIOMETRIC METHOD

A. Extremely sensitive; wide range of linearity
B. Quick
C. Can use undiluted tissue samples
    1. Radiometric method limits dilution-induced dephosphorylation and decarbamylation.
    2. An example comparison of the spectrophotometric vs. radiometric method for measuring AChE in carbamate-treated tissue.

V. CONCLUSIONS AND RECOMMENDATIONS

CHOLINESTERASE DATA COLLECTED FROM BIOASSAYS PERFORMED FOR HAZARD EVALUATIONS

Speaker: Karen L. Hamernik, Ph.D.
Office of Pesticide Programs
United States Environmental Protection Agency

I. Introduction
   A. Project purpose, scope, and participants
   B. Project goals
      1. Examine cholinesterase (ChE) method reliability
         a. Identify methods/protocols used
         b. Analyze ChE data variability
         c. Attempt to identify factors contributing to variability observed
      2. Build ChE historical control database

II. How Studies Containing ChE Data Were Selected For Analysis

III. How Studies Were Analyzed
   A. Summarization and comparison of methods, modifications, protocols, and procedures used
   B. Data collection, statistical treatment, and summarization

IV. Parameters Examined For Potential Contribution to Data Variability
   A. Results in control animals
      1. Species/strain
      2. Sex
      3. Age
      4. Type ChE measured (plasma, erythrocyte, brain)
      5. Method related-
         a. Protocol, laboratory Standard Operating Procedure (SOP), method modifications
         b. Instrumentation
         c. Sample collection
         d. Sample processing
   B. Variability in data from animals treated with an organophosphorus agent or a carbamate
   C. Other considerations
      (Intra- and interlaboratory differences in absolute ChE activity measurements)

Cholinesterase Assay Strawman Protocol
by Brian Dementi
U.S.E.P.A. Office of Pesticide Programs

I. INTRODUCTION

II. CHOLINESTERASE ASSAY PROTOCOL

a. Scope

Methodology for cholinesterase activity in plasma, erythrocytes, brain and other tissue, as derived from Ellman, additional journal publications and industrial SOPs

b. Assay Procedure

1. Laboratory equipment, reagents, etc.
2. Present draft procedure with item by item review of parameters for consideration by Workgroup

c. Specimen Collection/Preparation

Review technical aspects of the obtaining,preparation,storage, etc. of biological samples

d. Reporting of Results

e. Statistical Analysis of Data

EXISTING GUIDELINES FOR ASSESSING CHOLINESTERASE INHIBITION CAUSED BY OPs AND CARBAMATES

I. BRIEF HISTORY

A. 1959: FDA - Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics (ed. 1975)
2 species: rat and dog
blood: plasma and erythrocytes
10 males/10 females per dose
pre-dose measurements: 5 weekly determinations
dosing: levels from maximum tolerated to "no effect level"
enzyme measurements: beginning of test period at one and two weeks and then biweekly up to 12 weeks in 5 rats of each sex per group
post-dose measurements: same 5 M/F at 1 and 4 weeks after treatment

NOTE: " when plasma and erythrocyte provide the most sensitive index of cumulative effect, the response in dog has approached very closely that in the human." 4M/4F per dose; use each as their own control; 5 weekly pre-dose levels on each dog; during test, measure at 1 and 2 weeks then biweekly for duration of 90 day study.

B. 1972: EPA - Proposed Toxicology Guidelines
2 species: rat and dog
tissues: plasma and rbc
brain at termination
test duration: rat - 90 days and 2 years
dog - 90 days or 6 months
number of animals: rat - 15 M/F (90 day); 25-50 M/F (2 yr)
dog - 4M/4F
dosing: 3 levels from effect to no-effect level, plus control
enzyme measurements: 3, 6, 12, 18 and 24 months (rat)
3 and 6 months (dog)

C. 1975: EPA - Guidelines for Registering Pesticides in U.S. (Proposed)
Neurotoxicity: "The rat or dog is appropriate for demonstrating acetylcholinesterase inhibition."

D. 1978: EPA - Proposed Guidelines for Registering Pesticides in the U.S.; Hazard Evaluation: Humans and Domestic Animals
2 species: rat and dog
tissues: plasma and rbc
brain at termination
test duration: 90 days (rat); 6 months (dog)
number of animals: rat - 10 M/10F
dog - 6 M/6 F
dosing: 3 levels plus control, from no-effect to effect

enzyme measurements: twice before, twice during and at termination. In dogs, serial determinations may be useful to provide a time course of development of inhibition, extent of inhibition, and recovery from inhibition (e.g. after removal from treated diet). Dogs should be fasted 1 day prior to obtaining blood samples.

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test duration: 24 - 30 months (rat)
number of animals: at least 8/sex/group (out of 50M/50F)
dosing levels: 3 plus control
enzyme measurements: before dosing, every 6 months during and at termination.

E. 1982 and 1984 (Revised): EPA - Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Humans and Domestic Animals

2 species: rat and dog
tissues: not stated
duration: 90 day
number of animals: rat - 10 M/10F
dog - 4M/4F
dosing levels: 3 plus control
enzyme measurements: rats - termination
dogs - beginning, monthly intervals or midway through the test and at termination

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testing duration: rat - 12 -24 months
dog - 12 months
number of animals: rat - 10 M/10F out of 20M/20F
dog - 4M/4F
dosing levels: 3 plus control
enzyme measurements: at least 3 times; beginning, middle and end

F. 1991: EPA - Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Human and Domestic Animals Addendum 10 - Neurotoxicity

Delayed Neurotoxicity of OP Substances Following Acute and 28 Day Exposures
Study Conduct: Biochemical Measures - (A) NTE Assay. "Depending on the duration of acute signs as an indication of the disposition of the test material, the time for sacrifice for NTE and AChE assessment may be chosen at a different time to optimize detection of effects."
(B) AChE Measures. "Assay of acetylcholinesterase in the brains of the same birds shall also be performed."
References. Ellman, G.L. Biochem. Pharmacol. 7:88-95 (1961).

OVERVIEW OF TESTING GUIDELINES

DOCUMENT	PRE-DOSE	PERIODIC MEASURES	POST-DOSE MEASURES	TISSUES	METHOD
1959 FDA	5 weekly	1, 2 wks biweekly for 12 wks	1 and 4 weeks	pl, rbc	N.S.
1972 EPA GL	N.S.	3, 6, 12, 18 and 24 months (rat)	N.S.	pl, rbc, brain (term)	N.S.
		3, 6 mos. (6 mo. dog)	N.S.	pl, rbc, brain (term)	N.S.
1978 EPA GL	2 X before	90 day 2 X during and term	N.S.	pl, rbc, brain (term)	N.S.
	Before	24-30 Month every 6 mos. and term.	N.S.	pl, rbc, brain (term)	N.S.
1982/84 EPA GL	N.S.	90 day term. (rat); begin, monthly or midway, term (dog)	N.S.	N.S.	N.S.
		1-2 yr begin, midway, term.	N.S.	N.S.	N.S.
1991 EPA GL	Neurotox (Hen)	Term.	N.S.	brain	Ellman (1961)

RESEARCH NEEDS: ONGOING AND PROPOSED

Hugh A. Tilson, Neurotoxicology Division, Health
Effects Research Laboratory, U.S. EPA
Research Triangle Park, North Carolina

I. INTRODUCTION

A. Cholinesterase Inhibitors in the Environment

B. Recent Meetings on Cholinesterase Inhibitors
   1. June 1988, Risk Assessment Forum Colloquium
   2. May 1989, FIFRA SAP Open Meeting
   3. September 1989, Joint FIFRA SAP/SAB Open Meeting
   4. May 1991, OPP/HERL Briefing on Research Needs

II. IDENTIFIED RESEARCH NEEDS

A. Definition of Adverse Effect
   1. Use of cholinesterase levels in blood, brain
   2. Regional differences in brain
   3. Different esterase forms
   4. Use of neurobehavioral endpoints

B. Appropriate Surrogate for Human
   1. Critical evaluation and analysis of species
   2. Different species of rodents
   3. Different strains of rats

C. Developing Organism
   1. Brain acetylcholinesterase and development
   2. Relative sensitivity of developing organism

D. Repeated Exposure
   1. Tolerance to some but not all effects
   2. Residual effects following cessation of exposure

E. New Issues
   1. Accelerated aging and delayed onset neurodegeneration
   2. Ocular toxicity
   3. In vitro neurotoxicology

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
OFFICE OF PESTICIDE PROGRAMS
HEALTH EFFECTS DIVISION

CHOLINESTERASE METHODOLOGIES WORKSHOP DECEMBER 3-5 1991
SHERATON CRYSTAL CITY HOTEL, ARLINGTON, VIRGINIA

NEW OPPORTUNITIES IN CHOLINESTERASE TESTING PARADIGMS

DAN WOLF HANKE, PH. D.

OUTLINE

I. Opening remarks.

II. Recombinant human acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).

Generation and expression of the cDNA clones according to Mona Soreq *et al*.

- Use of synthetic oligodeoxynucleotide probes to search cDNA libraries originating from fetal and adult brain to identify cDNA clones for AChE and to search cDNA libraries originating from fetal and adult liver to identify cDNA clones for BChE.
- Generation of multiple copies of the AChE and BChE cDNAs using the polymerase chain reaction (PCR).
- In vitro transcription of the synthetic mRNAs from the AChE and BChE cDNAs
- Translation of the synthetic mRNAs encoding for AChE and BChE in microinjected Xenopus oocytes to generate and to characterize the recombinant AChE and BChE.
- Mass expression of the AChE and BChE synthetic mRNAs in bacteria- Escherichia coli- to generate large quantities of the recombinant AChE and BChE.

III. AChE from fetal bovine serum (FBS).

Affinity chromatography preparation of electrophoretically pure AChE according to BP Doctor *et al*.

- Cytotoxic serum, unusable for tissue culture, is mixed with sepharose attached to procainamide, which is the ligand binding the anionic site of AChE.

- Pack the mixture onto a column and elute the AChE off the column with decamethonium.

- The collected AChE is assayed and electrophoresed to establish specific activity and purity respectively.

IV. Suggested in vitro ChE screening paradigm using pure human recombinant AChE and BChE as well as AChE from FBS

- Set standard enzyme activity control rate of reaction (slope of curve for absorption plotted against time) that is linear for at least 15 min regardless of assay method.

- Set standard negative enzyme control with DFP or physostigmine at 20 % inhibition or use discrete increments, e.g., 20 %, 50 %, and 75 % inhibition.

- Test pesticide against the appropriate negative control at the same concentration(s). Result will be an immediate measure of ChE toxicity relative to the negative control. Also will have ability to immediately differentiate between the pesticide toxicity relative to each specific ChE, which is comparatively more difficult to do from a blood sample containing both ChEs thereby requiring selective inhibitors.

- May decide to stop here at this point if the relative toxicity of the pesticide is "too great" to warrant testing in vivo. Otherwise, continue on with the paradigm and determine next the rate constants, K_d, k_1, and k_2, for pesticide inhibition of each ChE. Inferences can be made from the rate constants whether acute toxicity may be involved.

- If the pesticide toxicity to the ChEs is "significant" then test oximes as antidotes for accidental poisoning and for use on labels.

- Run animal studies after the previous step (or in parallel from the outset). The in vitro data can help to prioritize an order for testing an array of pesticides in animals based on their toxicity to the pure ChEs.

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
CHOLINESTERASE METHODOLOGIES WORKSHOP 3-5 DECEMBER 1991
NEW OPPORTUNITIES IN CHOLINESTERASE TESTING PARADIGMS
DAN WOLF HANKE, PH. D.

V. Advantages and disadvantages of pure recombinant human ChEs and purified AChE from fetal bovine serum for an in vitro pesticide screening paradigm.

A. Advantages.

- o relatively inexpensive.
- o quick and easy to do- same day results.
- o reproducible to within 1-5 % of respective controls, since the ChEs are pure, and there are no interfering sample impurities.
- o the ChEs can be stable for at least one year when stored properly.
- o can use human ChEs to screen for direct human exposure to pesticides and food consumption of pesticide residues, etc.
- o can use the bovine ChE to screen for non food pesticide uses in or around cattle (dairy and/or beef) or in animal feed, etc.
- o rate constants may be used to estimate acute toxicity.
- o inhibited-ChE oxime-reactivation screening can identify antidotes for labeling purposes, etc.

B. Disadvantages.

- o availability on contract and commitment to quantity.
- o overall cost of screening program will be higher with the additional cost on the front-end, which may or may not be recovered down-the-line.