Office of Pesticide Programs
Science Policy on

The Use of Data on Cholinesterase Inhibition for Risk Assessments of Organophosphorous and Carbamate Pesticides

August 18, 2000

Office of Pesticide Programs
US Environmental Protection Agency
Washington DC 20460

TABLE OF CONTENTS

EXECUTIVE SUMMARY

The Office of Pesticide Programs (OPP) published a policy statement on the use of data on cholinesterase inhibition (and other events associated with cholinergic effects related to nervous system function) in human health risk assessment of certain classes of pesticide chemicals for review by the FIFRA Scientific Advisory Panel (SAP) in 1997 and for public comment in 1997 and 1998 (US EPA, 1997b). The 1997 science policy document described the approaches OPP would employ in assessing the potential for human health hazard from the cholinergic effects on nervous system function following exposure to cholinesterase-inhibiting pesticides.

The 1997 policy document has been reorganized and revised, taking into consideration, as appropriate, comments offered by the public, the SAP, other EPA offices and other government agencies. As did the 1997 policy, this revised science policy emphasizes the weighing of all relevant evidence when selecting endpoints for the hazard assessment of anticholinesterase pesticides. This "weight-of-the-evidence" review, conducted on a case-by-case, chemical-by-chemical basis, is accomplished by performing an integrative analysis after assessing all the individual lines of evidence (including all available data on cholinesterase inhibition in all compartments -- central nervous system, peripheral nervous system, red blood cells, and plasma -- as well as data on clinical signs, symptoms and other physiological or behavioral effects). Weighing of the evidence must include considerations of many factors, including the adequacy of study protocols; quality of data; number of studies on each endpoint; dose-dependency of responses; time course and duration of effects; and similarities or differences of responses observed in all the species, strains, and sexes tested for each duration and route of exposure evaluated.

In a weight-of-the-evidence assessment of cholinesterase-inhibiting substances, acetylcholinesterase inhibition in the nervous system is viewed as a key event in the mechanism of toxicity of these compounds and an important critical effect to consider in the hazard assessment. Evaluations of the cholinergic effects (i.e., physiological and

behavioral changes and measures of cholinesterase inhibition in the central and peripheral nervous systems) caused by exposure to the cholinesterase-inhibiting organophosphorous and carbamate pesticides provide direct evidence for characterizing potential human health hazard. Because of likely differences in both the chemicals' and the cholinesterases' pharmacodynamic properties, measures of cholinesterase inhibition in both the central and peripheral nervous systems are important for a thorough evaluation of potential hazard. However, direct measurement of cholinesterase activity in peripheral nervous system tissues are rarely available at the present time. When these data are not available, as a matter of prudent science policy protective of human health, EPA will treat cholinesterase inhibition in the blood as a surrogate measure for the peripheral nervous system in animals and for both the peripheral and central nervous systems in humans. Information from blood cholinesterase inhibition data is considered to provide important insights into potential hazard. Red blood cell (RBC) measures of acetylcholinesterase (AChE) are generally preferred over plasma measures of cholinesterase activity because data on red blood cells may provide a better representation of the inhibition of the neural target enzyme, acetylcholinesterase. OPP, however, may use plasma cholinesterase inhibition data under certain circumstances, such as if red blood cell data are insufficient, of poor quality, or unavailable; if there is a lack of dose-dependency for the red blood cell acetylcholinesterase inhibition; or, if the dose responses for inhibition of plasma cholinesterase more closely approximate those for AChE inhibition in the nervous system than do the dose responses for RBC acetylcholinesterase inhibition.

It should be noted that the present policy provides guidance *only* on how to deal with data as they relate to the cholinergic endpoints associated with nervous system *function* following exposure to organophosphorous and carbamate pesticides. This scope is consistent with all earlier descriptions of Agency assessment approaches as well as that of other organizations with regard to the evaluation of cholinesterase-inhibiting substances (e.g., WHO JMPR (1990,1999), DPR-CalEPA (1997) and other national authorities such as Canada's PMRA (Franklin, 1999). When applying the weight-of-the-evidence approach for selecting critical effect(s) for derivation of a

reference dose (RfD) or concentration (RfC), however, the entire toxicological data base on a pesticide must be evaluated (i.e., there also must be consideration of endpoints not related to the cholinergic consequences of anticholinesterase activity, for instance, liver or developmental toxicity or carcinogenicity). It is possible that, for one or more of the exposure scenarios being evaluated, the non-cholinergic effects will be identified as critical or co-critical, and they may become a more appropriate basis for deriving RfDs or RfCs.

Finally, OPP policy documents are meant to be "living documents," that is, they are open to periodic updating and revision to reflect advances in the science. Thus, this policy, too, will be updated to incorporate important new scientific knowledge as it becomes available. For example, the routine availability of data on acetylcholinesterase activity in the peripheral nervous system may allow for refinements in the hazard assessment approach for anticholinesterase chemicals. Also, as knowledge increases about the potential roles of the different cholinesterases in the developing organism, particularly as they impact the development of the nervous system, it may allow for refinements in evaluating the potential differential sensitivity and susceptibility of the young versus adults. In fact, a substantial research effort has been, and continues to be, made to determine what roles acetylcholine-, butyrylcholine- and other esterases may play in the development of the nervous system and in cell growth, proliferation and death in other tissues. OPP encourages further discussion of the possible implications of the research findings, both for future research planning and for the Agency's regulation of cholinesterase-inhibiting pesticides.

LIST OF ABBREVIATIONS

Scientific Terms:

ACHE	Acetylcholinesterase
BMD	Benchmark Dose
BuChE	Butyrylcholinesterase
LOAEL	Lowest-Observed-Adverse-Effect Level
MF	Modifying factor
NOAEL	No-Observed-Adverse-Effect Level
PoD	Point of Departure
RBC	Red Blood Cell (or erythrocyte)
RfC	Reference Concentration
RfD	Reference Dose
UF	Uncertainty Factor

Organizational Terms:

DPR-CalEPA	Department of Pesticide Regulation-California Environmental Protection Agency
FIFRA SAP	EPA's FIFRA Scientific Advisory Panel
ILSI	International Life Sciences Institute
PMRA Canada	Pesticide Management Regulatory Agency-Canada
NRC/NAS	National Research Council-National Academy of Sciences
OPP	Office of Pesticide Programs
SAB	EPA's Science Advisory Board
SAP	EPA's FIFRA Scientific Advisory Panel
TRAC	Tolerance Reassessment Advisory Committee
WHO/FAO JMPR	World Health Organization/Food and Agricultural Organization Joint Meeting on Pesticide Residues

ORGANIZATION OF POLICY DOCUMENT

This science policy document describes the approaches that the Office of Pesticide Programs (OPP) employs when evaluating data on cholinesterase inhibition and other cholinergic effects related to nervous system function that are the consequences of acetycholinesterase inhibition (i.e., physiological or behavioral changes) in assessing the potential hazard following exposure to organophosphorous or carbamate pesticides that inhibit cholinesterase.

- ❑ Section 1(**Introduction)** presents a very brief description of the Agency's general approach to non-cancer risk assessment. This chapter also includes a brief introduction to the biology of the cholinergic nervous system, description of cholinesterase enzymes and their distribution, the inhibition of acetylcholinesterase as a mechanism of toxicity for cholinesterase-inhibiting pesticides, and the consequences of acetylcholinesterase inhibition in the body.

- ❑ Section 2 (**Historical Background**) presents the history that has led to the development of this policy document.

- ❑ Section 3 (**Identification of the Toxicological Endpoints for Assessment of Cholinesterase Inhibitors**) describes the scientific rationale for selection of endpoints for anticholinesterase pesticides, their toxicological significance and relevance to hazard assessment.

- ❑ Section 4 (**Weight-of-the-Evidence Analysis for Selection of Critical Effects**) describes the weight-of-the-evidence approach that is used to select the critical effect(s) for the risk assessment of anticholinesterase pesticides.

- ❑ Section 5 (**Conclusions**) provides a summary of the key elements of the weight-of-the-evidence approach that OPP uses when evaluating anticholinesterase organophosphorous and carbamate pesticides.

- ❑ Section 6 **(References)** contains the literature citations and other references used as source material for the policy document.

1. INTRODUCTION

The purpose of this document is to set forth the principles and procedures, including a weight-of-the-evidence approach, used by OPP for the selection of appropriate endpoints for assessing potential hazards to humans exposed to anticholinesterase pesticides. In addition, this science policy document also describes science policy approaches specific to effects related to cholinesterase inhibition that will be used to address inadequacies in data or lack of knowledge. The Agency's policy which addresses the potential for pre- and postnatal effects and the completeness of databases with respect to toxicity and exposure as they relate to infants and children when conducting risk assessments and making regulatory decisions regarding the setting of tolerances (residues in food) under the 1996 Food Quality Protection Act can be found in the draft guidance document entitled "The Office of Pesticide Programs' Policy on Determination of the Appropriate FQPA Safety Factor(s) for Use in the Tolerance-setting Process" (US EPA, 1999b).

1.1 RISK ASSESSMENT FRAMEWORK

Regulatory decision making in EPA is described as consisting of two major steps--risk assessment and risk management–which are closely related but different processes. Risk assessment defines the potential for adverse effects[1] to occur in individuals or populations, while risk management weighs risk reduction alternatives and integrates the risk assessment with social, economic, and other factors, as appropriate. The Agency uses the paradigm put forward by the National Research Council of the National Academy of Sciences in 1983 and modified in 1994 (NRC/NAS, 1983; 1994) that defines and organizes risk assessment into four phases: hazard identification, dose-response assessment, exposure assessment, and risk

[1]Adverse effects include alterations from the baseline that diminish an organism's ability to survive, reproduce, or adapt to the environment. Neurotoxicity is defined as an adverse change in the structure or function of the central and/or peripheral nervous system following exposure to an agent (US EPA 1998a).

characterization. Risk assessment for noncancer effects including those addressed in this policy is generally based on identifying a no-observed-adverse-effect-level (NOAEL) or calculating a benchmark dose (BMD) for a critical effect[2], which is usually determined from laboratory animal studies, for use as a Point of Departure (PoD) when deriving a reference dose (RfD) or reference concentration (RfC). The point of departure (PoD) is generally defined as a point estimate of an empirically-measured or modeled dose or exposure level that is used as the "jumping-off" point for extrapolation to exposure levels below those tested, where actual human exposures are actually likely to be occurring. The PoD can be a dose at which no effects are found or a dose level which is associated with some percent of response relative to the control or baseline level of response. The PoD is divided by one or more uncertainty factors (UF) or modifying factors (MF). The UFs (typically 3- or 10-fold in magnitude) reflect uncertainties inherent in the extrapolation from laboratory animal species to humans (the interspecies UF), in the variations in sensitivity among members of the human population (the intraspecies UF), for the use of subchronic rather than chronic data (the subchronic to chronic UF), the use of a lowest-observed-adverse-effect level (LOAEL) rather than a NOAEL (the LOAEL to NOAEL UF), and the comprehensiveness and quality of the database available, i.e., whether or not all potential endpoints of concern are identified and evaluated in acceptable studies (the database UF). A modifying factor may be used to address scientific uncertainties in the principal study used for RfD/C derivation which are not explicitly addressed by the other standard Ufs.

The result of dividing a PoD by the appropriate uncertainty factors and/or modifying factor is a reference dose (RfD) for oral or dermal exposures–or reference concentration (RfC) for inhalation exposure(s). The RfD or RfC is defined as an estimate, within an order of magnitude, of exposure assumed to be without appreciable risk for adverse noncancer health effects. In the risk characterization step, the RfD and

[2]"Critical effect" is defined in EPA's Integrative Risk Information System (IRIS) as "the first adverse effect, or its known precursor, that occurs as the dose rate increases." Information on the derivation of reference doses or reference concentrations can be found at the IRIS web site http://www.epa.gov/ngispgm3/iris/rfd.htm.

RfC values are compared to potential or known exposure levels. Risk characterization also fully describes the nature and extent of the risks posed, and how well the data support the conclusions, including a discussion of the limitations and uncertainties involved. Sometimes, because of these limitations and uncertainties, further data may be collected to reduce the uncertainties and refine the risk assessment.

The Agency has acknowledged that the historical approach to defining a NOAEL and calculating RFDs and RfCs has limitations (see USEPA, 1994; 1995; 1996). In response, the Agency has developed draft guidance on an alternative method–the Benchmark Dose (BMD) Approach (USEPA, 1996). The BMD is defined as the statistical lower confidence limit on the dose producing a predetermined level of change in response compared with the background response. A BMD is derived by fitting a mathematical model to the dose-response data.[3] The Agency is still gaining experience with BMD analyses and has not yet formally finalized standard operating procedures. OPP, however, will use the BMD approach for derivation of RfDs and RfCs to the extent possible.

1.2 BIOLOGY AND TOXICOLOGY OF CHOLINESTERASE INHIBITION

Acetylcholine plays an important role in the functioning of the nervous system.[4] Acetylcholine is a neurotransmitter which enables chemical communication to occur between a nerve cell and a target cell. This target cell may be another nerve cell, muscle fiber or gland. Upon stimulation, the nerve cell releases acetylcholine into the synapse (or space) between the two cells. This released acetylcholine binds to

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[3]Draft benchmark software for hazard endpoints is currently available at http:\www.epa.gov\ncea\bmds.

[4]Summary reviews of the cholinergic components of the nervous system and of the toxicity of anticholinesterase chemicals can be found in several chapters of two widely-available textbooks (Ecobichon (1996), Hoffman, et al., (1996), and Taylor (1996a, 1996b)) and in Dementi (1997) which served as a technical support document to the 1997 policy document.

receptors on a target cell, thereby passing the signal on to that nerve cell, muscle or gland. The end result of the stimulation of cholinergic pathway(s) includes, for example, the contraction of smooth (e.g., in the gastrointestinal tract) or skeletal muscle, changes in heart rate or glandular secretion (e.g., sweat glands) or communication between nerve cells in the brain or in the autonomic ganglia of the peripheral nervous system. Cholinergic pathways innervate virtually every organ in the body, including the brain and peripheral nervous system.

There are two major divisions of the nervous system, both of which contain cholinergic pathways that may be affected by cholinesterase-inhibiting chemicals:

- ❏ the peripheral nervous system, consisting of neuromuscular junctions in skeletal muscle, and tissues of the autonomic nervous system, consisting of ganglia of the sympathetic and parasympathetic nervous systems, smooth muscles, cardiac muscle, and glands; and

- ❏ the central nervous system, consisting of brain and spinal cord.

The distribution of cholinergic receptors in the central nervous system and the peripheral nervous system is not uniform (Brimijoin, 1992). For example, certain brain regions of the mature organism are rich in cholinergic neurons (e.g., the striatum, hippocampus, cerebral cortex), while other regions are less well innervated and have less cholinesterase activity (e.g., cerebellum). There are two major types of cholinergic receptors -- muscarinic and nicotinic -- and there are several subtypes of each. These receptor types also are differentially distributed in different regions of the central and peripheral nervous systems, thus contributing to the complexity of effects that may occur.

Acetylcholinesterase (AChE) is found in cholinergic neurons, in the vicinity of synapses, and in other, non-neural tissues. It is highly concentrated at the neuromuscular and other neuroeffector junctions. It is the enzyme that breaks down acetylcholine and terminates its action in the synapses between neurons and between neurons and muscle fibers or glands. Inhibition of AChE leads to an accumulation of acetylcholine and a prolongation of the action of acetylcholine at the nerve-nerve, nerve-muscle or nerve-gland interface. Peripherally, the accumulation of acetylcholine can result in cholinergic responses such as smooth muscle contractions (e.g., abdominal cramps), glandular secretions (e.g., sweating), skeletal muscle twitching, and, at higher concentrations, flaccid paralysis. In addition, there may be centrally-mediated effects on learning, memory and other behavioral parameters. Thus, the inhibition of AChE potentially results in a broad range of adverse effects, having an impact on most bodily functions, and depending on the magnitude and half-life of an exposure dose, these effects can be serious, even fatal.

Effects caused by AChE inhibition may be a result of action on neurons in the central nervous system and/or the peripheral nervous system. Access of chemicals to the central nervous system and the peripheral nervous system may be different because of differences in pharmacokinetic properties of these two compartments (e.g., differences in absorption, distribution, metabolism, elimination). These differences may be due to chemical specific characteristics as well as the characteristics of the exposed organism (e.g., degree of maturation of the blood-brain barrier). The pattern of effects seen may also depend upon factors such as the pharmacodynamic characteristics (i.e., binding potency, rate of reversal) of the cholinesterase-inhibiting chemical and the molecular form of cholinesterase with which it is interacting (e.g., see Scarsella, et al., 1979).

Butyrylcholinesterase (BuChE) is similar in structure to AChE, but it is encoded by a separate gene. BuChE, which is synthesized primarily in the liver and found in plasma and other tissues, is generally distinguished from AChE by BuChE's slower rate of hydrolysis of acetylcholine, by function and by localization using histochemical

techniques after subjecting the experimental model to inhibitors which selectively block the activity of one but not the other enzyme (Taylor and Radic, 1994). Furthermore, the binding affinity of anticholinesterase chemicals for each enzyme can differ among these substances (Silver, 1974; Taylor and Radic, 1994). Both enzymes are present during development of the nervous system, with the ratios of one to the other changing substantially over time and with location (Hoffman, et al., 1996). While no neurological function has been shown definitively for BuChE in the developing or mature nervous system, the BuChE present in the plasma will catalyze the hydrolysis and inactivation of ingested esters from plant sources (e.g., cocaine and related synthetic local anesthetics (Hoffman, et al.,1996) and neuromuscular blocking agents such as succinylcholine (Taylor, 1996b). Likewise, there is no known physiological or biochemical function for erythrocyte acetylcholinesterase (Brimijoin, 1992; Dementi, 1997).

As discussed later, the blood cholinesterase enzymes are regarded, as a matter of policy, as surrogate measures of neuronal cholinesterase activity. Of the two common blood elements measured, red blood cells (RBC) contain AChE exclusively, while the ratio of AChE to BuChE in plasma varies widely among humans, dogs, and rats, the species in which these measures are most typically made for risk assessment and regulatory purposes. While human plasma is overwhelmingly BuChE, the plasma of dogs and rats contains both AChE and BuChE (Scarsella, 1979; Edwards and Brimijoin, 1983).

The question of whether, and, if so, how, BuChE plays a role in the development and/or functioning of the nervous system still awaits resolution. Research has been, and continues to be, conducted to determine if butyrylcholinesterase plays a role in nervous system morphogenesis (development) and function, and whether, and if so, how, butyryl- and/or acetylcholinesterase and other esterases play a more general role in cell growth and death, including in carcinogenesis. In addition, the dose response relationships attendant to acetylcholinesterase's function(s) in the development of the nervous system remain to be described and compared with those of the endpoints

currently used in the evaluation of nervous system function. OPP is preparing a brief summary of the available literature on the role of the cholinesterases (and, perhaps, other esterases) in these areas. OPP also is preparing a series of questions to serve as a starting point for discussion for addressing whether or not this information may justify further revisions to the present policy. This effort is being conducted separately from the revision of the current policy document.

2. HISTORICAL BACKGROUND

2.1 OPP's HISTORICAL APPROACH

Cholinesterase inhibition and the cholinergic effects (i.e., the physiological or behavioral changes) caused by organophosphorous and carbamate pesticides have long been endpoints that OPP has used in assessing potential human health hazards. For well over a decade, OPP has regarded data showing cholinesterase inhibition in brain, RBC, or plasma, and data on physiological or behavioral changes as critical effects (i.e., effects that should be considered for use in the derivation of an RfD or RfC). OPP has used statistical significance, rather than a fixed percentage of response from baseline, as the primary, but not exclusive, determinant of toxicological and biological significance in selecting Points of Departure (e.g., NOAELs or LOAELs or Benchmark Doses). This approach treats cholinesterase activity data like most continuous endpoints (*i.e.,* graded measures of response such as changes in organ weight, hormone levels or enzyme activity), where no fixed generic percentage of change from the baseline is considered to separate adverse from non-adverse effects (US EPA, 1995). OPP believes that a fixed percentage for describing adversity that would apply to all cholinesterase-inhibiting pesticides or to all compartments (i.e., blood, central nervous system, peripheral nervous system) cannot be determined realistically or scientifically justified. Each data set must be judged on its own merits, consistent with the weight-of-the-evidence approach that OPP is implementing. The use of uncertainty factors and the use of statistical significance are consistent with Agency practice for all non-cancer, systemic toxicity endpoints.

OPP's Reference Dose Tracking Report (US EPA, 1997a) lists chronic Reference Doses for over 50 chemicals based in whole, or in part, on cholinesterase inhibition. There are, however, many more than 50 risk assessments that make use of this endpoint in acute and chronic dietary exposure/risk assessments and in other, non-dietary scenarios representing both short-term and intermediate-term exposure(s).

2.2 REVIEWS OF PROPOSED AGENCY/OPP SCIENCE POLICY POSITIONS

Prior to 1997, one internal Agency colloquium (US EPA, 1988) and two public Science Advisory Board (SAB)/Scientific Advisory Panel (SAP) meetings (SAB/SAP, 1990; 1993) considered draft Agency guidance on the use of cholinesterase data in risk assessment. An additional SAP/SAB review in 1992 of a proposed reference dose for aldicarb also addressed the issue of cholinesterase inhibition as an endpoint in risk assessment (SAB/SAP, 1992). Each of these reviews yielded somewhat different perspectives and recommendations, based in part on somewhat differing proposed policies, but primarily on differing points of view of each peer review group. The area of greatest divergence among these reports and in their recommendations involved the interpretation and use of blood measures of cholinesterase inhibition, particularly in plasma, for deriving reference doses. Some reviewers and panels placed less (or no) reliance on plasma measures of cholinesterase inhibition and/or less reliance on red blood cell measures of AChE inhibition as a critical effect than OPP traditionally has placed on each. EPA has never finalized guidance on this topic for use Agency-wide.

In 1997, OPP published its own policy statement on the use of data on cholinesterase inhibition for risk assessments, accompanied by case studies illustrating the application of this policy and a review of pertinent literature on cholinesterase inhibition prepared by OPP staff for public comment and SAP review (US EPA, 1997b; Dementi, 1997). In 1998, as part of the OPP review process for science policy issues agreed upon in conjunction with the Tolerance Reassessment Advisory Committee (TRAC), OPP again made the 1997 policy paper available for broader public comment (US EPA, 1998b).

The 1997 OPP policy statement described a weight-of-the-evidence approach for use when evaluating the data on cholinesterase inhibition and its consequent potential adverse cholinergic effects. The SAP expressed support for the use of such an approach (SAP, 1997). Briefly, the SAP stated that:

> ... the weight of evidence approach is indeed reasonable and justified on the basis of the available scientific data so long as these data are derived from rigorous experiments with standardized methods and proper controls. In particular, this approach allows flexibility to weight heavily inhibition in non-target tissue when the overall toxicologic context suggests that other approaches pose danger of serious risk from overexposure. (Emphasis added in original.)

The 1997 policy paper also proposed that the differences of opinion with respect to the use of blood measures in risk assessment could be reduced or resolved by the collection of peripheral nervous system tissue measurements of AChE inhibition in animal studies which might serve instead of the blood measures as critical effects for use in hazard assessment. The 1997 SAP concluded that the use of blood measures "is readily justified if the discrepancy between blood cholinesterase and functional endpoints is not too great" and recommended that data on AChE inhibition in the peripheral nervous system be collected. On these points, the SAP also noted that:

- There was unanimous support for the notion that, under SOME circumstances, measurement of SOME blood-borne cholinesterases would be appropriate to consider in establishing RfDs for anticholinesterases....(Emphasis added in original.)

  and

- Measured inhibition of cholinesterase activities in any of the blood fractions is best regarded as an imperfect mirror of enzyme inhibition in the true target tissues...

OPP subsequently asked the International Life Sciences Institute (ILSI)/Risk Science Institute to convene a workgroup to help further define the feasibility and details for collecting these data. This workgroup's report concluded that it was currently feasible to measure AChE inhibition in the peripheral nervous system (Mileson, et. al., 1999b). The ILSI workgroup further concluded, "Methods and techniques currently available are adequate to characterize the AChE activity in the peripheral nervous system, but additional studies would help to improve these methods."

3. IDENTIFICATION OF THE TOXICOLOGICAL ENDPOINTS FOR ASSESSMENT OF CHOLINESTERASE INHIBITORS

This Section explains the science policy decisions and rationale specific to the evaluation of the various cholinergic effects on nervous system function caused by anticholinesterase pesticides. This rationale forms the basis of the weight-of-the-evidence approach described later in Section 4. The general principles, including definitions of key terms, and approaches used by OPP and EPA for evaluating the neurotoxic potential of environmental agents can be found in the Agency's Guidelines for Neurotoxicity Risk Assessment (US EPA, 1998a).

This Section is organized around conclusions followed by a rationale addressing three key types of endpoints generally assessed currently for cholinesterase-inhibiting pesticides:

1) evaluations of physiological and behavioral/functional effects;

2) measures of acetylcholinesterase inhibition in the neural tissues (i.e., brain and peripheral nervous system); and

3) measures of cholinesterase inhibition in the blood (i.e., red blood cells and plasma).

3.1 EVALUATION OF EFFECTS ON CHOLINERGIC FUNCTIONS

3.1.1 CONCLUSIONS

- Clinical signs/symptoms[5] in humans and behavioral or physiological effects in humans and animals provide the most direct evidence of the potential adverse consequences of human exposure to anticholinesterase pesticides.

- Effects observable in humans[6] can cover a broader range than those that can be observed in animal studies, including psychological complaints, cognitive complaints and other subjective effects, and performance measures of learning and memory. Human studies following either deliberate or inadvertent exposure, nevertheless, are currently quite limited in the scope of the evaluations made and scale of the measurements used. As for animal studies, it is possible that one or more effects of concern may be occurring but measures for their evaluation were not or could not be incorporated in the study design. Also, the generally small numbers of subjects may limit the power of the study to detect effects of concern.

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[5]A "symptom" is defined as a condition which is a departure from normal function reported by the person experiencing and reporting that condition (e.g., headache, nausea). A "clinical sign" is an objectively-measured effect (e.g., heart rate, blood pressure) indicative or suggestive of a condition for an individual (human or animal) observed and recorded by another such as a physician.

[6] EPA is currently reviewing its policy concerning human studies with respect to ethical and scientific standards for their acceptability and use in risk assessments, particularly with respect to decisions under FQPA. The Agency neither requires nor encourages the conduct of human hazard identification studies to detect potential adverse effects of pesticides. EPA has held two meetings of a joint SAP/SAB (Science Advisory Board) panel (December 1998 and November, 1999 (US EPA, 1998c,1999d)) on the ethical elements of this issue. The Panel is expected to issue its report in the Summer of 2000.

- Evaluation of physiological and behavioral changes (i.e., functional data) in animal studies also are limited in terms of the scope of effects assessed and the measurements employed. As with human studies, it is possible that one or more effects of concern may be occurring but procedures for measuring these effects were not or could not be incorporated in the study design, leaving the possibility of a false negative.

- Because of the limited range of measures of behavioral and physiological effects evaluated historically, functional data obtained from human and animal studies should not be relied on solely, to the exclusion of other kinds of pertinent information, when weighing the evidence for selection of the critical effect(s) that will be used as the basis of the RfD or RfC.

3.1.2 RATIONALE

Many of the adverse acute and longer-term effects of anticholinesterase organophosphorous pesticides that have been observed in humans were described by Morgan (1989) and updated by Reigart and Roberts (1999):

> Most commonly reported in humans are headache, nausea, and dizziness. Anxiety and restlessness are prominent. Worsening may result in muscle twitching, weakness, tremor, incoordination, vomiting, abdominal cramps, diarrhea. Often prominent are sweating, salivation, tearing, rhinorrhea, and bronchorrhea. Blurred and/or dark vision, and excessive contraction of the pupil of the eye (miosis) may also be seen. Tightness in the chest, wheezing and productive cough may progress to frank pulmonary edema. Bradycardia may progress to sinus arrest, or tachycardia and hypertension. Confusion, bizarre behavior, and toxic

psychosis may occur. In severe poisonings, toxic myocardiopathy, unconsciousness, incontinence, convulsions, respiratory depression and death may be seen. Repeated absorption, but not enough to cause acute poisoning may result in persistent anorexia, weakness, and malaise.

As noted, many of the effects described above may be seen after acute exposures of humans to anticholinesterase pesticides. There also are case reports describing long(er)-term effects following acute high-level exposures. Little information exists describing effects following long(er)-term, low-level exposures to humans. Several cholinesterase inhibiting chemicals from both the organophosphorous and carbamate classes have been used and/or are being explored for usefulness in the treatment of certain neuromuscular diseases and neurocognitive disorders such as Alzheimer's disease (Taylor, 1996a; Standaert and Young, 1996). The literature describing the results of clinical trials and imposition of treatment regimens for these drugs may be useful in providing insights into the nature (both therapeutic and adverse) of human responsiveness to these substances.

Increasing levels of exposure generally result in progressively more serious effects, although the exact pattern of effects differs among anticholinesterase chemicals and may be influenced by the age of the patient, genetic differences, drug interactions, and other factors. Different cholinesterase-inhibiting chemicals may, and generally do, produce different spectra of clinical signs and behavioral effects. This complexity, in part, may arise from differences between the absorbed chemicals in distribution between the central and peripheral nervous systems and differential binding in those nervous system compartments, or differential interactions with the two major types of cholinergic receptors (*i.e.*, muscarinic and nicotinic receptors). The nature and temporal pattern of effects also depends on the magnitude, duration, and frequency of exposure, as well as whether metabolic activation is needed. Perhaps one third of the

effects caused by anticholinesterase chemicals (*e.g.*, headache, confusion, tremor, and convulsions) can be attributed primarily to effects on the central nervous system (Minton and Murray, 1988). For many effects, however, it is difficult to distinguish whether they are centrally or peripherally mediated or both.

For any pesticide, including cholinesterase inhibitors, OPP may require different toxicology studies in laboratory animals, depending upon the use and exposure patterns of the substance. Not all of these studies include the requirement for measurement of cholinesterase activity or the effects occurring as a consequence of its inhibition. Those that do or could are noted with an asterisk (*). The key studies required for a food-use pesticide of conventional chemistry are:

- Acute oral, dermal, and inhalation lethality tests in mammals;
- Acute or subchronic (90-day) delayed neurotoxicity study in hens;
- Acute and subchronic (90-day) neurotoxicity screening battery in rats*, which includes:
  - Functional observational battery, which is a set of structured observations outside the home cage, including assessments of autonomic signs, pupillary response to light or pupil size, arousal, reactivity, posture and gait, grip strength, limb splay, and simple sensory reflexes (e.g., tail pinch and a sudden sound);
  - Automated motor activity;
  - Histopathology of neural tissue from animals prepared by *in situ* perfusion;

- Responses to visual or proprioceptive (i.e., sense of body position or awareness of pressure) stimuli are optional, but not commonly done.

! 21-Day or subchronic (90-day) dermal toxicity study in mammals*;

! Subchronic (90-day) inhalation study in mammals* (if appropriate on basis of anticipated human route of exposure);

! Two chronic toxicity studies*, one in the rat and one in the dog;

! Two prenatal developmental toxicity studies*, one in a rodent and one in a non-rodent species; and

! Two-generation reproduction study in rodents*; and

! Developmental neurotoxicity study in rats*, which includes, in pups:

- detailed observations, developmental landmarks, motor activity, auditory startle reflexes, learning and memory test, and neuropathology on postnatal days 11 and 60
- detailed observations for neurological effects also are made in dams

While they never have been a part of EPA's data requirements and, thus, there are no EPA testing guidelines for them, human hazard identification studies on some pesticides have been submitted by the chemical's sponsor(s) and, in the past, prior to the passage of FQPA, considered for use in risk assessments. These hazard identification studies typically are designed to identify no-effect levels for ChEI-associated enzyme activity and, sometimes, for some clinical effects. Although many of these human hazard identification studies with cholinesterase inhibitors are acute (i.e., single dose) in their exposure duration, some have incorporated short-term (e.g., 4-10 day) or longer (e.g., 21-28 day) repeat dosing. Measures of cholinesterase inhibition in

either whole blood (which is a mixture of plasma and RBCs), or separately in RBCs and plasma are usually included. Sometimes, reporting of some clinical symptoms and signs are included; in a few cases, objective physiological measures, such as blood pressure, pulse rate or temperature, have been reported.

Human hazard identification studies can be designed to detect more effects in addition to blood enzyme inhibition (e.g., mild sweating and nausea) compared to animal studies, due to self-reporting of complaints, including sensory, cognitive, and psychological effects. Formal evaluations (by interview or test), however, are very uncommon as are measurements of physiological parameters like heart function (e.g., heart rate and blood pressure) and breathing rate. More sophisticated neurobehavioral test batteries, such as intelligence tests or simple memory tests, used in epidemiological studies (for example, Anger, et al., 1996), are rarely, if ever, used in human hazard identification studies of cholinesterase-inhibiting organophosphorous and carbamate pesticides.

The reports of certain kinds of animal studies include detections of overt clinical signs, including many of the autonomic signs, and motor effects, such as tremors. In the rodent neurotoxicity screening battery studies, the data are gathered systematically by observers unaware of treatment. The measurements of effects are defined quantitatively, albeit, usually on an ordinal scale (*e.g.*, +1, +2). Valid screening studies also include automated and quantitative measures of motor activity, grip strength, and limb splay, though changes in these measures are not a distinguishing characteristic of cholinesterase inhibition. EPA's test guidelines for the neurotoxicity screening batteries were published in 1991. OPP has received data from these neurotoxicity screening studies on many of the anticholinesterase organophosphorous and carbamate pesticides. Of the roughly 30 effects that may occur following acute exposures as listed by Morgan (1989) and updated by Reigart and Roberts (1999), perhaps one third would not be seen in routine animal studies, or even in the neurotoxicity screening battery, as they are currently designed, especially the sensory, cognitive, and psychological effects. Thus, because of the limitations in the study

design and conduct of both human and animal studies, OPP may not understand fully the profile of effects of concern that may result from exposure to the cholinesterase-inhibiting pesticides.

3.2 NERVOUS SYSTEM CHOLINESTERASE INHIBITION

3.2.1 CONCLUSIONS

- Inhibition of acetylcholinesterase in the nervous system (both central and peripheral) is generally accepted as a key component of the mechanism of toxicity leading to adverse cholinergic effects. The inhibition of this enzyme provides direct evidence of potential adverse effects. Interference with the timely deactivation of neuronal or neuroeffector acetylcholine results in the protraction of the actions of acetylcholine at these sites, which in turn results in adverse cholinergic effects. Because the inhibition of acetylcholinesterase is a key event that can lead to adverse effects, data showing this response provide valuable information in assessing potential hazards posed by anticholinesterase pesticides.

- Measures of acetylcholinesterase activity in both central and peripheral nervous tissues are important for a full assessment of the potential for hazard because the enzyme and each chemical may have different pharmacokinetic and pharmacodynamic properties in each compartment of the nervous system.

- The relationships between the functional effects and changes in acetylcholinesterase activity in both nervous system compartments often are difficult to characterize with existing data for a variety of reasons (e.g., development of tolerance, heterogeneity of cholinergic pathways including the molecular form(s) of AChE present at each location, limited data on the regional distribution of acetylcholinesterase, the time course of inhibition in each region, and limited evaluation of functional effects).

3.2.2 RATIONALE

The inhibition of acetylcholinesterase is a key step in the mechanism of toxicity of certain organophosphorous and carbamate pesticides (Mileson, et al, 1998; Mileson, 1999a; US EPA, 1999a, 1999c), and, therefore, measures of cholinesterase inhibition represent a critical biochemical biomarker of potential adverse effects. Inhibition of acetylcholinesterase in the central nervous system is considered to be an indicator of an adverse effect. Nonetheless, reductions in neural AChE activity may not always be accompanied by overt clinical signs or symptoms because, for example, the critical functions of those specific neurons may not be sufficiently evaluated to detect related changes or tolerance may have developed. The time at which potential functional effects are evaluated may also contribute to an apparent lack of concordance between functional effects and the neurochemical effects (i.e., cholinesterase inhibition). Based on these, and, perhaps, other factors, it is difficult to determine, with accuracy or consistently, the degree of cholinesterase inhibition that will cause specific physiological or behavioral changes. Thus, OPP considers a treatment-related decrease in brain or peripheral tissue AChE activity, in itself, toxicologically important. Data showing such a decrease are appropriate for use as a critical effect for the derivation of RfDs and RfCs, as well as for characterizing potential human hazards.

Historically, data on central nervous system AChE inhibition have come from single or repeated exposure animal studies, in which whole brain homogenates are assayed at one or two time points. For the past several years, more detailed measurements of brain AChE inhibition have been required. These requirements, as part of the neurotoxicity screening battery, or as separate studies, have sought to characterize the time course of inhibition in plasma, RBCs, and brain, including in specific brain regions, after acute and 90-day exposures. Even so, most of the existing data sets will generally contain measures only of whole brain AChE activity, but not usually regional brain measurements, or time-course data, particularly following acute exposures. The lack of regional brain measures appears to be a limitation, given that the distribution of cholinergic pathways and the concentration and molecular form of AChE in different brain regions is not homogenous. Thus, whole brain measurements of AChE inhibition may reveal little or no change in activity while masking significant changes in specific brain regions associated with particular cholinergically-mediated functions (e.g., the hippocampus and memory).

Unfortunately, measures of AChE inhibition in peripheral neural tissues or neuroeffector junctions are rare. AChE inhibition data from the peripheral nervous system potentially have unique value because many of the adverse signs and symptoms associated with exposure to anticholinesterase pesticides (e.g., diarrhea, excess salivation) are a result of effects on the peripheral nervous system. Because of the potential pharmacokinetic differences between the central and peripheral nervous compartments, measures of AChE activity in both of these systems are important for the full assessment of chemicals on the nervous system. Certain chemicals may have equivalent access to a specific compartment, in both degree and rate of interaction. On the other hand, there are others for which the rate of access to, and concentration in, peripheral tissues is far greater than in the central nervous system. These patterns could shift with longer term exposures.

Although AChE inhibition data in peripheral nervous system tissues have not been required in toxicological studies submitted to EPA and, at the moment, no standard protocol exists for the generation of such data, OPP indicated in 1997 that the collection of these measures potentially could become an alternative to the use of blood cholinesterase inhibition measures in animal studies in the hazard and risk assessment process. As discussed earlier, the SAP (SAP, 1997) and an expert panel of ILSI (Mileson, et al. 1999b) have stated that it is feasible to measure AChE inhibition in peripheral nervous system tissues. The 1997 SAP report asserted, "it is important that joint efforts be mounted to evaluate AChE inhibition in the peripheral neural tissues *per se* and in the neuroeffector junctions." The SAP expressed the view that it is technically feasible to routinely conduct AChE assays on the peripheral nervous system, while recognizing the difficulties involved. The SAP further suggested that skeletal muscles, heart, lung, salivary glands, diaphragm, and autonomic ganglia (*e.g.*, superior cervical ganglia) be considered as appropriate tissues to examine. The SAP considered that standardized and reproducible dissection and homogenization of tissue, assays with minimal tissue dilution, selection of the most relevant tissue targets, and standardization of tissue storage conditions were the most important technical issues to resolve when measuring AChE activity in the peripheral nervous system. Work is underway in EPA's National Health and Environmental Effects Laboratory (NHEERL) to develop and standardize protocols for assaying enzyme activity in various peripheral tissues (e.g., see Marshall, et al., 1999).

3.3 BLOOD CHOLINESTERASE INHIBITION

3.3.1 CONCLUSIONS

! Inhibition of blood cholinesterases (i.e., plasma and red blood cell) is not itself an adverse effect, but may indicate a potential for adverse effects on the nervous system. As a matter of science policy, blood cholinesterase data are considered appropriate surrogate measures of potential effects on peripheral nervous system acetylcholinesterase activity in animals, for CNS

acetylcholinesterase activity in animals when CNS data are lacking and for both peripheral and central nervous system acetylcholinesterase in humans.

- As such, blood cholinesterase inhibition data are considered appropriate endpoints for derivation of reference doses or concentrations when considered in a weight-of-the-evidence analysis of the entire database on a single pesticide or on two or more pesticides assigned to a common mechanism of toxicity group, where acetylcholinesterase inhibition is the common mechanism of toxicity.

- Red blood cell measures of acetylcholinesterase inhibition, if reliable, generally are preferred over plasma data. Since the red cell contains only acetylcholinesterase, the potential for exerting effects on neural or neuroeffector acetylcholinesterase may be better reflected by changes in red blood cell acetylcholinesterase than by changes in plasma cholinesterases which contain both butyrylcholinesterase and acetylcholinesterase in varying ratios depending upon the species. This conclusion rests on data showing that chemicals may have significantly different interactions with AChE and BuChE, including their affinities for binding with the enzymes.

- Although RBC acetylcholinesterase data are generally preferred, in some cases, reliance on measures of RBC may not be appropriate because of methodological issues concerning blood measures of cholinesterase activity. When making weight-of-the-evidence judgments concerning the selection of RBC versus plasma measures of cholinesterase inhibition as endpoints for derivation of reference doses or concentrations, it is critical to consider all aspects of the information database, including the adequacy of the study protocol, quality of the data, dose-dependency of the responses, as well as available data on measures of brain acetylcholinesterase inhibition and functional effects.

- Plasma contains both butyrylcholinesterase and acetylcholinesterase in varying ratios depending upon the species. The separate characterization of RBC and

plasma measures of cholinesterase inhibition provides an additional means of measuring effects. Additionally, having separate RBC and plasma data allow for more informative animal-to-human comparisons.

3.3.2 RATIONALE

As a biomarker of exposure, blood cholinesterase inhibition can be correlated with the extent of exposure. As discussed earlier, there is often a direct relationship between a greater magnitude of exposure and an increase in incidence and severity of clinical signs and symptoms as well as blood cholinesterase inhibition. In other words, the greater the exposure, the greater the cholinesterase inhibition in the blood and the greater the potential for an adverse effect to occur. Both plasma and RBC measures of cholinesterase inhibition also provide:

- pharmacokinetic evidence of absorption of the pesticide and/or its active metabolite(s) into the bloodstream and systemic circulation; and

- pharmacodynamic evidence of binding to AChE, the neural form of the target enzyme, or to plasma BuChE, an enzyme similar in structure to AChE

Because the interaction with AChE is widely accepted as a key event of the mechanism of toxicity for anticholinesterase pesticides, inhibition of this cholinesterase in the blood creates the presumption that a chemical also is causing inhibition of neural AChE. Chemicals are absorbed into the blood and transported to the peripheral nervous system. Pharmacokinetically, the blood compartment and the peripheral nervous system are "outside of" the central nervous system, i.e., separated from the CNS by the blood-brain barrier. Thus, blood measures of cholinesterase activity are viewed as a better surrogate for the effects on AChE in the peripheral nervous system than are enzyme changes in the central nervous system. Because data on AChE inhibition in the peripheral nervous system have rarely been gathered in animals, blood cholinesterase inhibition measures are generally the only information available to assess the potential of chemicals to inhibit AChE in the peripheral nervous system. In

human studies, blood cholinesterase inhibition measures serve as surrogates for effects in both the central and peripheral nervous systems because neither of these neural tissues is available for evaluation directly. As discussed earlier, evaluations of clinical signs and symptoms have limitations, and thus should not be relied on solely, to the exclusion of other data. Therefore, blood cholinesterase inhibition data are considered appropriate endpoints for derivation of reference doses or concentrations when considered in a weight-of-the-evidence analysis of the entire database on a single pesticide or on two or more pesticides assigned to a common mechanism of toxicity group, where acetyl- cholinesterase inhibition is the common mechanism of toxicity.

The usefulness of collecting blood cholinesterase inhibition data (both RBC and plasma) is illustrated further by its use in monitoring workers for occupational exposures (even in the absence of signs, symptoms, or other behavioral effects). Blood cholinesterase inhibition (RBC and/or plasma) is considered as providing a sufficient basis for removing workers from the exposure environment, given the assumption that if one were to protect against enzyme inhibition, one would protect against effects of graver concern. For example, the California Department of Health Services (CDHS) requires monitoring of agricultural workers who have contact with highly toxic organophosphorous or carbamate compounds (EPA Toxicity Category I or II pesticides; $LD_{50} \leq 500$ mg/kg in rats)(CDHS, 1988). CDHS removes workers from the workplace whose plasma levels show 40% or greater cholinesterase inhibition from baseline, or whose red blood cell cholinesterase levels show 30% or greater inhibition. Workers may not return until their cholinesterase values return to within 80% of baseline. The World Health Organization (WHO) also has guidelines with the same RBC action levels (i.e., 30% or greater inhibition), and considers plasma inhibition of 50% of baseline to

indicate a "toxic" decrease (Fillmore and Lessinger, 1993). Fillmore and Lessinger also reviewed the California program and found that "The relative risk of pesticide poisoning was increased in workers whose initial baseline plasma levels were low, or if their levels had already dropped to 60-80% of their baseline previously in the season."

Although a pesticide's effect(s) on either RBC and plasma cholinesterase activity is considered to provide information on its potential to inhibit AChE in the nervous system, data from RBCs, which contain AChE exclusively, may better reflect neuronal AChE inhibition than data from the plasma, which is a variable mixture of butyrylcholinesterase and acetylcholinesterase. As discussed earlier, acetylcholinesterase is the enzyme involved in the mechanism of toxicity for the cholinergic effects of anticholinesterase pesticides. Although BuChE is somewhat similar in structure to AChE, BuChE is nevertheless sufficiently different in important ways which often result in it having binding affinities to anticholinesterase agents as well as other characteristics that are quite different from those of acetylcholinesterase (Silver, 1974; Taylor and Radic, 1994). The composition of plasma cholinesterases varies widely among humans, dogs, and rats, the species for which these measures are most typically made. Human plasma is overwhelmingly BuChE with a ratio of BuChE to AChE of 1,000:1 (Edwards and Brimijoin, 1983). In dogs, there is a little more than 10% acetylcholinesterase in plasma with a ratio of BuChE to AChE of 7:1 (Scarsella *et al.*, 1979). In rats, plasma contains approximately 50% or more of AChE with a BuChE to AChE ratio of 1:3 in males and 2:1 in females (Edwards and Brimijoin, 1983). While it is technically possible to ascertain the contribution of each ChE to the level of inhibition in plasma, this type of data is rarely available. Thus, the relationship between blood measures of AChE and BuChE or other factors is usually not known. For these reasons, a treatment-related decrease in plasma cholinesterase activity, viewed in isolation, provides less insight into the potential of a chemical to cause neural AChE inhibition than do data on RBC AChE inhibition.

Historically, there have been technical difficulties with the measurement of the inhibition of plasma and RBC cholinesterase(s), particularly for the latter (see Wilson, et al., 1996). Although in recent years there have been improvements in blood measures of cholinesterase activity, it is important to consider carefully the methodological issues that may affect the accuracy and variability of the data when assessing the effects of pesticides on cholinesterase activity in blood. There are many methods available for measuring blood cholinesterase activity. The colorimetric method, based on the Ellman reaction, is considered a reliable method when performed properly, and is commonly used for measuring plasma and RBC cholinesterase activity (Ellman, *et al.*, 1961; US EPA, 1992; ASCP, 1994). While well suited to the measurement of cholinesterase inhibition induced by organophosphorous pesticides, the Ellman method may underestimate cholinesterase activity in both plasma and RBC following carbamate exposure because of the relatively unstable binding of the carbamate esters to the acetylcholinesterase. The radiometric method may be better suited for measuring carbamate-inhibited cholinesterase (Johnson and Russell, 1975; Wilson, et al., 1996). The refinement of measurement methods continues in NHEERL. If, and when, the conduct of future animal studies on pesticides includes the measurement of peripheral nervous system acetylcholinesterase activity, in addition to central nervous system measures, then less reliance can be placed on the use of blood measures as critical effects for the derivation of RfDs/RfCs in the risk assessment process. However, as noted above, blood measures will, in any case, continue to provide important information in characterizing human hazard because they still will provide a means of animal-to-human comparison of cholinesterase inhibition. As noted above, work on standardizing methods for measuring acetycholinesterase activity in the peripheral nervous system is underway. OPP/EPA expects that the standardization and use of these methods will result in a database that improves the scientific understanding of the risks of cholinesterase-inhibiting compounds.

4. WEIGHT-OF-THE-EVIDENCE ANALYSIS FOR SELECTION OF CRITICAL EFFECTS

The present science policy has been prepared considering the comments received from the SAP and the public in 1997 and during the public comment period in 1998. This revised policy continues to embrace the weight-of-the-evidence approach of considering all relevant data in an integrative manner that was described in the 1997 OPP document (US EPA, 1997). This revised policy expands the discussion of the approach and clarifies the weight-of-the-evidence approach by describing more explicitly under what conditions and how plasma and/or RBC cholinesterase data would be considered. The policy also re-emphasizes the potential usefulness of collection of peripheral neural data on AChE inhibition to reduce reliance on the surrogate blood measures.

OPP is using the weight-of-the-evidence approach described here to analyze individual studies as well as the complete database on a pesticide when selecting critical effects for hazard assessment. The primary objective of the weight-of-the-evidence analysis for anticholinesterase pesticides is to select Points of Departure (PoDs) (i.e., NOAELs, LOAELs, or benchmark doses) for critical effects to be used in the calculation of RfDs, RfCs or margins of exposure (MOE) for all of the routes and durations of exposure appropriate for a pesticide given its use and exposure patterns when, after review of the entire toxicological database, it is concluded that the cholinergic effect(s) induced by the substance being evaluated do, in fact, represent the critical effect(s). Briefly, the weight-of-the-evidence approach includes consideration of all available data on:

- clinical signs and other physiological and behavorial effects in humans and animals;

- symptoms in humans;

- central nervous system acetylcholinesterase inhibition;
- peripheral nervous system acetylcholinesterase inhibition;
- red blood cell acetylcholinesterase inhibition; and
- plasma cholinesterase inhibition (BuChE in humans; mixed AChE/BuChE in animals).

A comparison of the pattern of doses required to produce physiological and behavioral effects and cholinesterase inhibition in different compartments is conducted. In addition to these parallel analyses of the dose-response information, comparisons of the temporal aspects (e.g., time of onset and peak effects and duration of effects) of each relevant endpoint are made. This analysis should be done for each relevant route and duration of exposure (e.g., acute, intermediate and/or chronic exposures) for each available species/strain/sex of animals. Furthermore, the potential for differential sensitivity/susceptibility of adult versus young animals (i.e., effects following perinatal or postnatal exposures) to anticholinesterase chemicals should be assessed. These analyses should be conducted in the context of the adequacy of the protocols used and the quality of the available data. Based on this weight-of-the-evidence analysis for an anticholinesterase pesticide, OPP may select as the critical effects any one or more of the behavioral and physiological changes or enzyme measures listed above.

Although clinical signs/symptoms, physiological and behavioral changes are considered very important for characterizing an adverse effect in humans, these endpoints are not given disproportionate emphasis or relied on solely, or even always necessarily preferred, in selecting critical effects for risk assessment because the evaluations of such endpoints have limitations. Comprehensive measures of AChE activity in nervous system tissues, particularly as they may reflect age-related differences, are considered very important and are given considerable prominence in the weight-of-the-evidence analysis for selection of critical effects because, as

discussed earlier, acetylcholinesterase inhibition is considered a key event in the mechanism of toxicity for the cholinesterase-inhibiting organophosphorous and carbamate pesticides and a substantial body of literature exists which links enzyme inhibition with a broad range of adverse effects. Thus, data on cholinesterase inhibition may be viewed as predictors of potential adverse responses mediated via cholinergic pathways and may be used instead of, or in the absence of, data on clinical signs and symptoms, and other physiological and behavioral effects. Direct measures of AChE inhibition in the neural target tissues, (i.e., central and peripheral nervous systems) are preferred. However, when such data are missing or inadequate, they would obviously receive less weight in the analysis. In these circumstances, measures of cholinesterase inhibition in the blood (plasma and/or RBC) are viewed as reasonable surrogates for the peripheral nervous system given that the blood is the pharmacokinetic compartment into which chemicals are absorbed and transported to the peripheral nervous system. In animals, data on blood cholinesterase inhibition also are considered important companion data for central nervous system AChE inhibition data, even though the brain constitutes a different pharmacokinetic compartment. As noted earlier, blood measures (both plasma and RBC) of cholinesterase activity in human studies must serve as surrogates for enzyme activity in both central and peripheral nervous systems, in light of the lack of availability of data on these parameters. As discussed in Section 3.3, within the blood compartment, RBC AChE data, if reliable, are generally preferred over plasma data. Even though plasma contains a mixture of AChE and BuChE, plasma cholinesterase data should be evaluated and considered in the parallel analyses as described below. Also, there may be certain situations where plasma cholinesterase inhibition may be selected as the critical effect for the risk assessment.

Evaluation of the statistical and toxicological significance of the study results and application of uncertainty factors follows the Agency's established procedures for derivation of an RfD or RfC and the principles articulated in the FQPA 10X Safety Factor policy. A description of the strengths, weaknesses, and limitations of the database is included; this evaluation may lead to the identification of data needed to refine the data base and the risk assessment. Any residual concerns (i.e., significant

uncertainties) are accommodated for when making the FQPA 10X Safety Factor determination.

Practically, the weight-of-the-evidence analysis may be viewed as having several steps: first, the individual studies are evaluated; second, all studies in the database and their relationship to one another are examined in an integrated manner; and lastly, the critical effects are selected for risk assessment and additional data needs identified. Below is a more detailed discussion of these steps.

4.1 ANALYSIS OF INDIVIDUAL STUDIES

For a full evaluation of an anticholinesterase pesticide, the important elements of a study should include:

- Evaluations of physiological and behavioral effects;
- Measures of central nervous system acetylcholinesterase activity (in animal studies) (often these will be whole brain measures rather than measures in specific brain regions)
- Measures of peripheral nervous system acetylcholinesterase inhibition (in animal studies) (rarely available at the present time);
- Measures of RBC and plasma cholinesterase inhibition.

First, each study is critically evaluated. Results should be assessed in the context of both statistical *and* biological significance. No fixed percentage of change (e.g. 20% for cholinesterase enzyme inhibition) is predetermined to separate adverse from non-adverse effects. OPP's experience with the review of toxicity studies with cholinesterase-inhibiting substances shows that differences between pre- and post-exposure of 20% or more in enzyme levels is nearly always statistically significant and

would generally be viewed as biologically significant. The biological significance of statistically-significant changes of less than 20% would have to be judged on a case-by-case basis, noting, in particular, the pattern of changes in the enzyme levels and the presence or absence of accompanying clinical signs and/or symptoms. The study evaluation involves consideration of, among other factors: the adequacy of study protocol and design (e.g., experimental group size and characteristics (e.g., single gender or both), dose spacing, methods used for neurochemical and functional evaluations); whether pre-exposure data were obtained in the subsequently-exposed individuals (i.e. measures are taken before and after treatment in the same individual, then statistically analyzed as such) or a separate control group was used, and the general conduct of the study. The consistency of the findings within the study when repeated measures are taken, the dose-dependency of the responses, as well as the temporal aspects of effects (e.g., the time-of-onset, steady state, time-to-peak effects and the time until complete recovery) and the statistical significance of any differences measured between unexposed and exposed groups are to be examined before reaching any conclusions regarding biological significance. The relationship of the different effects seen to one another also should be considered in interpreting the findings. Following critical evaluation of the validity of the study, candidate points of departure (i.e., NOAELs, LOAELs, or benchmark doses) are identified or calculated.

4.2 INTEGRATIVE ANALYSIS OF THE DATA BASE

When evaluating the entire database and selecting an endpoint(s) as the critical effect(s) to serve as the PoD in the derivation of a RfD or RfC, parallel analyses of the dose-response (i.e., changes in magnitude of enzyme inhibition or of a different effect with increasing dose) and the temporal pattern of all relevant effects will be compared across all of the different compartments affected (*e.g.*, plasma, RBC, peripheral nervous system, brain), and for the functional changes to the extent the database permits. The overall adequacy of the test protocols and the quality of the data also are important elements of the analysis. The consistency (or, the lack thereof) of LOAELs, NOAELs, or BMDs for each category of effects (e.g., clinical signs, cholinesterase

inhibition in the various compartments, etc.) for the test species/strains/sex available and for each duration and route of exposure should be noted. If scientifically valid, reliable, and ethically appropriate to use, human data may be preferable to animal data because they preclude the need for extrapolation of results across species, avoiding the uncertainties attendant to this aspect of the risk assessment process. Confidence in the selection of an endpoint(s) for derivation of an RfD or RfC is enhanced by the factors described in Table 1. The findings for anticholinesterase pesticides will span a broad continuum, and their databases will range from those which are comprehensive and robust to those which are limited and of poor quality. Thus, end point selection and weight-of-the-evidence judgments must be made on a case-by-case basis. For example, often cholinesterase inhibition data in a single compartment may be inconsistent across studies involving the same species or strain. In some cases, large differences may be noted in the magnitude of cholinesterase inhibition in one compartment in comparison to all the other compartments. In other cases, there is no dose-effect relationship for cholinesterase inhibition in one or more compartments. Time course data for cholinesterase inhibition also are often limited. Brain measures of AChE activity are often limited to whole brain at termination of an animal study. So, a typical database for an anticholinesterase

Table 1. Factors to consider for selection of Points of Departure for anti-cholinesterase chemicals

Higher confidence:

- Methodologies employed are valid and reliable
- Sample processing is reliable and appropriate
- Reliable data are available for RBC, plasma, and brain cholinesterase inhibition as well as functional data in individual studies
- Responses are dose dependent, and NOAELs are apparent
- Time-to-peak effects and steady state are demonstrated
- Responses for each end point evaluated are consistent within multiple

pesticide will likely contain a number of inadequacies that can have a broad spectrum of influence from none to substantial on the selection of critical effects. It should be emphasized, however, that the lack of, or deficiency in, any one factor listed in Table 1 would not necessarily discount the usefulness of a study in selecting an endpoint for calculation of an RfD or RfC. Functional evaluations are limited in both human and animal studies. Therefore, as described earlier, measures of cholinesterase inhibition are included in the weight-of-the-evidence evaluation; the reliance on all relevant data is considered to be both scientifically sound and public health protective. Cholinesterase inhibition in the blood may occur at lower doses than other cholinergic effects (e.g., brain AChE inhibition, functional effects). The NOAEL or equivalent benchmark dose for RBC AChE inhibition and that for plasma and/or brain may not be the same. This could be due to methodological problems or to the different binding affinities of a pesticide to AChE compared to those for BuChE or to a number of other factors. As explained in Section 3.3, if the measurements of AChE inhibition in RBCs are considered methodologically sound, these data generally are preferred over plasma cholinesterase activity data as predictors of neural AChE activity, even if the plasma NOAEL/BMD is lower. However, if the RBC data are unreliable (e.g., questions exist about the methodology or there is no dose-dependency) or the dose response for inhibition of plasma cholinesterase more closely approximates that for AChE inhibition in the nervous system than does the dose response for RBC acetylcholinesterase inhibition, plasma cholinesterase inhibition may be the more prudent endpoint to use to represent the critical effect.

Occasionally, because of methodological difficulties or for other, poorly-understood reasons, empirical correlations between the doses that cause plasma and brain cholinesterase inhibition (in the same or other studies) may be stronger than those between the doses for RBC and brain enzyme inhibition.

The weight-of-the-evidence approach emphasizes the determination of the quality of the cholinesterase data, especially the RBC measures. Standard operating procedures for measuring cholinesterase activity have continued to evolve over the last decade (Wilson, et. al., 1996; Hunter, et. al., 1997); detailed information on the method(s) and procedures used for measurements of cholinesterase activity following treatment is important. The method used for carbamate pesticides is particularly important because the reliability of data on cholinesterase effects depends not only on the specific methodology used, but to a great extent on sample processing (given the readily reversible nature of the carbamylated AchE).

4.3 COLLECTION OF PERIPHERAL NERVOUS SYSTEM CHOLINESTERASE INHIBITION DATA

As discussed earlier, the 1997 FIFRA Scientific Advisory Panel endorsed collection of peripheral nervous system AChE data as being technically feasible and advised OPP that these data may be a better indicator of cholinergic effects than are blood cholinesterase measures. The ILSI Panel provided more technical guidance along with a number of recommendations for further studies to improve the methodologies, while, nonetheless concluding that such measures could be taken now (Mileson, et al., 1999b). OPP agrees that peripheral nervous system measurements from a suitable set of tissues could provide an alternative to blood measures. OPP, with ongoing technical and research support from NHEERL, will continue to support the development and validation of methodologies for measuring peripheral neural AChE inhibition. This was a major aspect of OPP's policy in 1997 and was endorsed by the 1997 SAP. Once a methodology is validated, data from such studies will be sought on a regular basis and used to supplement or replace blood measures which now serve as

a surrogate for the peripheral nervous system. In the interim, any data on peripheral tissues will be evaluated and incorporated into the risk assessment on a case-by-case basis. OPP strongly encourages the development of any data aimed at refining risk assessments based upon blood measures to be focused on peripheral nervous system measures of AChE. Additional data to differentiate between the acetylcholinesterase and butyrylcholinesterase in plasma, a procedure recommended by the SAP in 1997, would also be useful.

Additional studies to provide data on metabolism, pharmacokinetics and pharmacodynamics also may be useful to aid in the characterization of the cholinesterase inhibiting properties and potential hazard of organophosphorous and carbamate pesticides. To lessen the uncertainties inherent in route-to-route extrapolation, endpoint specific data could be collected on exposure routes of interest, such as cholinesterase inhibition following dermal exposure.

5. CONCLUSION

The elements of the weight-of-the-evidence evaluations used for selecting toxicity endpoint(s) are summarized in Table 2. Weight-of-the-evidence judgments must be sound and supported by the data on the individual pesticides. The risk assessor should provide a hazard characterization that summarizes the endpoint data that are available for consideration, discusses the strengths, limitations and uncertainties of the data, and describes how well the data supports the conclusions. The rationale for selection of the critical effect(s) must be clearly articulated in this characterization.

Table 2. Elements of the Weight-of-the-Evidence Approach

OPP is using a weight-of-the-evidence approach to analyze individual studies and the overall database on an organophosphorous or carbamate cholinesterase-inhibiting pesticide to select the appropriate critical effect(s) for hazard assessment.

Based on this weight-of-the-evidence analysis, OPP may select as critical effects any one or more of the following functional or biochemical measures:

- physiological and behavioral effects in humans and animals;
- central or peripheral nervous system tissue acetylcholinesterase inhibition;
- red blood cell acetylcholinesterase inhibition;
- plasma cholinesterase inhibition (butyrylcholinesterase in humans;a mixture of acetylcholinesterase and butyrlcholinesterase in animals).

Because functional evaluations have limitations, measures of acetylcholinesterase inhibition are considered in the weight-of-the-evidence for selection of critical effects. Although data on acetylcholinesterase inhibition in neural tissues are preferred, peripheral nervous system data are rarely available in animals, and in humans neither peripheral nor central nervous system data are typically available. Blood measures can serve as surrogate information for projecting potential hazards, as a matter of science policy in protecting human health. Although plasma cholinesterase data may be used under certain circumstances, in general, red blood cell data, if available and reliable, are preferred over plasma data.

In selecting the appropriate endpoint(s) for hazard assessment, all available response data (i.e., all available cholinesterase enzyme activity and functional data) are evaluated for data quality, and analyzed for comparisons of dose-dependency, time to onset, steady state, time to peak effects, duration of effects within and across all test species/sexes for all durations and routes of exposure. Evaluation of statistical and toxicological significance, and application of uncertainty factors, follows established Agency procedures for the derivation of reference doses or reference concentrations. If limitations are identified in the database, additional data may be required to reduce any uncertainties and refine the hazard assessment

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