UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
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OFFICE OF THE ADMINISTRATOR
SCIENCE ADVISORY BOARD

EPA-SAB-EHC-93-011

Honorable Carol M. Browner
Administrator
U.S. Environmental Protection Agency
401 M Street, S.W.
Washington, D.C. 20460

Subject: Science Advisory Board/Scientific Advisory Panel's review of the Risk Assessment Forum's document *Guidance on the Use of Data on Cholinesterase Inhibition in Risk Assessment* (August, 1992).

Dear Ms. Browner:

Cholinesterase inhibition resulting from exposure to drugs and chemicals (especially carbamate and organophosphorus pesticides) has long been an issue of concern to EPA and others involved in assessing environmental health risks. EPA currently evaluates the risks from cholinesterase inhibitors using clinical effects, brain cholinesterase inhibition, and/or blood cholinesterase inhibition to define hazard and set Reference Doses (RfDs). This policy stems from the efforts of an EPA Technical Panel which reviewed the literature on cholinesterase inhibition and proposed some science policy positions (1988) and the comments of a Science Advisory Board/Scientific Advisory Panel Special Joint Study Group which reviewed the Agency's position in 1989.

In August, 1992, the Risk Assessment Forum prepared a new draft policy document addressing the key issues identified in the earlier review, with particular regard to their application to risk assessment. The Agency asked that a new Joint Committee of the Science Advisory Board and the Scientific Advisory Panel (SAB/SAP) review critically the Forum's new draft document; consequently, the Committee met on November 5, 1992 in Washington, DC. The primary issues addressed at this review, and the Committee's findings and comments follow below:

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a) *Does the document accurately represent the relevant data and constitute a credible analysis of the scientific information on cholinesterase inhibition?*

The Committee found that the positions presented in the draft document are, in general, well supported by the underlying scientific data, and considers it to be a credible document. As with any such undertaking, however, the Committee noted areas in which improvements could be made. In particular, the section of the document addressing red blood cell (RBC) measures of inhibition should be rewritten for clarity, and the document should be revised to stress the need for better studies on several critical areas -- the relevance of cholinesterase inhibition (erythrocyte, plasma and brain) measurements; methods to compare results of such methods among laboratories; and the subsequent use of these measurements as biomarkers of exposure or as correlates to data on clinical signs and symptoms. The Forum document also needs to consider the peripheral effects of anticholinesterases, in addition to the focus on the effects of these agents on the central nervous system. The Committee was also concerned that, in general, the EPA document did not give adequate weight to the problems associated with the inhibition of peripheral nervous system cholinesterase.

b) *Are the following Agency positions consistent with available scientific information:*

1) *Clinical effects associated with exposure to cholinesterase inhibitors can be used in risk assessment to define hazard and to calculate benchmark doses and RfDs.*

The Committee agrees that clinical effects associated with exposure to cholinesterase inhibitors can be used to establish benchmark doses and reference doses (RfD), but only in conjunction with other relevant toxicological information. The inclusion of biochemical data regarding cholinesterase inhibition in conjunction with these signs and symptoms is considered essential for the complete hazard evaluation for these compounds.

2) *Cholinesterase inhibition in plasma and in red blood cells constitutes a biomarker of exposure.*

We recommend that the Agency's policy continue to include the use of blood cholinesterase data in the risk assessment process. The Committee agrees that blood cholinesterase inhibition is a biomarker of exposure which offers crucial supporting data for confirming exposures and corroborating clinical signs.

3) *Statistically significant cholinesterase inhibition in brain tissue of animals can be used in risk assessment to define hazard and to calculate benchmark doses and RfDs.*

The Committee noted several problems with the use of cholinesterase inhibition in animal brain tissue as a means of defining hazard and calculating benchmark or reference doses. These issues will have to be resolved if brain cholinesterase inhibition data are to be used with confidence in regulatory decision-making.

4) *To date, analyses of studies of cholinesterase inhibition in plasma and in red blood cells do not provide information useful for evaluating potential hazards and risks in the nervous system. This finding justifies a new science policy against the use of blood cholinesterase inhibition data for risk assessment purposes.*

Extant animal studies (cited in the enclosed report) provide conflicting evidence on the issue of using RBC cholinesterase inhibition data by itself (i.e., in the absence of clinical symptoms) for risk assessment purposes. Measurement of plasma and RBC cholinesterase inhibition should not be used by itself, but should be used in conjunction with the clinical data.

5) *Should data emerge of a consistent predictive relationship between red blood cell cholinesterase and neurotoxicity, those data will be evaluated on a case-by-case basis to determine the utility of red blood cell acetyl cholinesterase inhibition in risk assessment to define hazard and to calculate benchmark doses and RfDs.*

The Committee recommends that EPA evaluate the possibility that an RfD could be set based on clinical signs and symptoms that would be associated with a

significant inhibition of cholinesterase occurring at a specified dose. The Committee also recommends that EPA continue research aimed at examining carefully the correlation of clinical signs and erythrocyte cholinesterase inhibition, particularly regarding the correlations with respect to dose, time, and linearity. Such studies will be very important in future decisions on the usefulness of erythrocyte cholinesterase inhibition in regulatory decision-making.

We appreciate the opportunity to review this document, and look forward to your response to the issues we have raised.

Raymond C. Loehr

Dr. Raymond C. Loehr, Chair
Science Advisory Board

Gary P. Carlson

Dr. Gary P. Carlson, Chair
Science Advisory Board/Scientific Advisory Panel
Joint Committee

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EPA

AN SAB REPORT: CHOLINESTERASE INHIBITION AND RISK ASSESSMENT

REVIEW OF THE RISK ASSESSMENT FORUM'S DRAFT *GUIDANCE ON THE USE OF DATA ON CHOLINESTERASE INHIBITION IN RISK ASSESSMENT* BY THE SAB/SAP JOINT COMMITTEE

NOTICE

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ABSTRACT

In August, 1992, the EPA Risk Assessment Forum prepared a new draft policy document addressing key issues in assessing the risks from cholinesterase inhibitors. A Joint Committee of the Science Advisory Board and the Scientific Advisory Panel reviewed the document on November 5, 1992 in Washington, DC.

The Committee found that the draft document is generally supported by the underlying scientific data. Improvements could be made in the material addressing red blood cell (RBC) inhibition and the document revised to stress the need for better studies on the relevance of cholinesterase inhibition (erythrocyte, plasma and brain) measurements; methods to compare measurement results methods among laboratories; and the use of these measurements as biomarkers of exposure and correlates to data on clinical signs and symptoms. The document should consider the peripheral effects of anticholinesterases.

The Committee agrees that clinical effects associated with exposure to cholinesterase inhibitors can be used to establish benchmark doses and reference doses (RfD), but only in conjunction with other relevant toxicological information. The Committee also recommends that the Agency's policy continue to include the use of blood cholinesterase data in the risk assessment process, and agrees that blood cholinesterase inhibition is a biomarker of exposure which offers crucial supporting data for confirming exposures and clinical signs.

EPA should evaluate the possibility that an RfD could be set based on clinical signs and symptoms associated with a significant inhibition of cholinesterase occurring at a specified dose. EPA should continue research to examine the correlation of clinical signs and erythrocyte cholinesterase inhibition.

KEYWORDS: Cholinesterase; cholinesterase inhibition; anticholinesterases; organophosphates; neurological; myopathy; pesticides.

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1. EXECUTIVE SUMMARY

The positions presented in the draft *Guidance on the Use of Data on Cholinesterase Inhibition in Risk Assessment* are, in general, well supported by the underlying scientific data, and the Committee considers it to be a credible document. As noted in the following report (section 3.2.5), however, the *Guidance* may misinterpret the findings reported in the studies of Kaplovitz (1984) and of Blick (1989) on red blood cell (RBC) measures of inhibition; this section of the document should be rewritten for clarity. The document should be revised to stress the need for better studies on the relevance of cholinesterase inhibition (erythrocyte, plasma and brain) measurements, better methods to compare results among laboratories, and the use of these measurements either as biomarkers of exposure or as correlates to data on clinical signs and symptoms. The focus of the document on the central nervous system should be broadened to consider the peripheral effects of anticholinesterases as well. The Committee was concerned that, in general, the EPA document did not give adequate weight to the problems associated with the inhibition of peripheral nervous system cholinesterase.

The Committee agrees that clinical effects (as defined in section 3.2.1 of this report) associated with exposure to cholinesterase inhibitors can be used to establish benchmark doses and reference doses (RfD), but only in conjunction with other relevant toxicological information. The Committee finds that the **sole** use of clinical signs of toxicity, or the lack thereof, (especially in long-term exposure studies) for the assessment of hazard and determination of reference or benchmark doses may not be justified. The inclusion of biochemical data regarding cholinesterase inhibition in conjunction with these signs and symptoms is considered essential for the complete hazard evaluation for these compounds.

We recommend that the Agency's policy continue to include the use of blood cholinesterase data in the risk assessment process. The Committee agrees that blood cholinesterase inhibition is a biomarker of exposure which offers crucial supporting data for confirming exposures and corroborating clinical signs.

The Committee noted several problems with the use of cholinesterase inhibition in animal brain tissue as a means of defining hazard and calculating benchmark or reference doses, stemming from our lack of knowledge about the most sensitive brain regions and their physiological functions. These issues will have to be resolved if brain

cholinesterase inhibition data are to be used with confidence in regulatory decision-making.

Extant animal studies provide conflicting evidence on the issue of using RBC cholinesterase inhibition data by themselves (i.e., in the absence of clinical symptoms) for risk assessment purposes. There are strong theoretical reasons why the correlation should exist for agents that traverse the blood-brain barrier. Although this is difficult to demonstrate empirically, it is nevertheless true, for such agents, that blood enzyme inhibition precedes (and therefore predicts) brain enzyme inhibition. In the absence of any other test of proven greater reliability, measurement of plasma and RBC cholinesterase inhibition should be retained in EPA's policy and used in conjunction with clinical data. The Committee recommends that EPA evaluate the possibility that an RfD could be set based on clinical signs and symptoms that would be associated with a significant inhibition of cholinesterase occurring at a specified dose.

The Committee also recommends that EPA continue research aimed at examining carefully the correlation of clinical signs and erythrocyte cholinesterase inhibition, particularly regarding the correlations with respect to dose, time, and linearity. Such studies will be very important in future decisions on the usefulness of erythrocyte cholinesterase inhibition in regulatory decision-making.

2. *INTRODUCTION*

2.1 Background

Cholinesterase inhibition resulting from exposure to drugs and chemicals (especially carbamate and organophosphorus pesticides) has long been an issue of concern to EPA and others involved in assessing environmental health risks. Questions exist as to which measures (e.g., inhibition in red blood cells, plasma, the brain, etc.) best correlate with neurotoxic effects and are most appropriate for use in hazard identification and risk assessment. The major issue has centered on the relevance of data on cholinesterase inhibition in either plasma or red blood cells in establishing a predictive or causative relationship with effects on the nervous system, both central and peripheral.

For some years EPA has been evaluating the risks from cholinesterase inhibitors using clinical effects, brain cholinesterase inhibition, and/or blood cholinesterase inhibition to define hazard and set Reference Doses (RfDs). In 1988, an EPA Technical Panel reviewed the literature on cholinesterase inhibition and proposed some science policy positions. External reviews of that paper confirmed the existence of areas of both controversy and of consensus (U.S. EPA, 1988). A Special Joint Study Group of the Science Advisory Board and the Scientific Advisory Panel reviewed the Agency's position in 1989, expressing concern with regard to using plasma and red blood cell inhibition as a measure of toxicity, especially in the absence of other effects, but concurring that inhibition was a biomarker for exposure and absorption (U.S. EPA, 1990). The Study Group also noted that the relationship between the degree of inhibition noted and toxicity remained unclear, because of the complexities of the dose and time relationship, and differences among individual chemicals.

The Risk Assessment Forum's present paper replaces the 1988 draft, and reflects Agency consideration of comments on that draft. In summary, the Agency continues its policy to recognize clinical effects of cholinesterase inhibition as biologically significant, and to use them to set Reference Doses (RfD). The Agency will also continue to use statistically significant inhibition of cholinesterase in brain, with or without accompanying clinical manifestations, to set RfDs. The Agency proposes changing its position on the use of red blood cell/plasma cholinesterase inhibition in risk assessment. Plasma and red blood cell inhibition data will be used as biomarkers of

exposure to cholinesterase inhibiting chemicals, but, except for special cases, the Agency will no longer define hazard or compute RfDs based on such data alone.

2.2 Charge

The Agency asked the Joint Committee of the Science Advisory Board and the Scientific Advisory Panel (SAB/SAP) to review critically the Risk Assessment Forum's document *Guidance on the Use of Data on Cholinesterase Inhibition in Risk Assessment*. Although EPA is interested in the Committee's comments on all aspects of the document, there is special interest in the Committee's response to the following specific questions:

a) The science policy position articulated in the Risk Assessment Forum document is based in part upon an analysis of scientific data bearing on cholinesterase inhibition. Does the document accurately represent the relevant data and constitute a credible analysis of the scientific information?

b) Are the following Agency positions consistent with available scientific information:

   1) Clinical effects associated with exposure to cholinesterase inhibitors can be used in risk assessment to define hazard and to calculate benchmark doses and reference doses (RfDs).

   2) Cholinesterase inhibition in plasma and in red blood cells constitutes a biomarker of exposure.

   3) Statistically significant cholinesterase inhibition in brain tissue of animals can be used in risk assessment to define hazard and to calculate benchmark doses and reference doses (RfDs).

   4) To date, analyses of studies of cholinesterase inhibition in plasma and in red blood cells do not provide information useful for evaluating potential hazards and risks in the nervous system. This finding justifies a new science policy against the use of blood cholinesterase inhibition data for risk assessment purposes.

5) Should data emerge of a consistent predictive relationship between red blood cell cholinesterase and neurotoxicity, those data will be evaluated on a case-by-case basis to determine the utility of red blood cell acetylcholinesterase inhibition in risk assessment to define hazard and to calculate benchmark doses and RfDs.

The Committee met on November 5, 1992, in Washington DC to address the issues noted above.

3. DETAILED FINDINGS

3.1 Analysis of Relevant Data on Cholinesterase Inhibition

Within the physical limitations and constraints of the document, the material presented in *Guidance on the Use of Data on Cholinesterase Inhibition in Risk Assessment* represents, in general, the critical scientific data and is credible. It presents a simple but accurate assessment of the role of cholinesterase and the problems involved with its inhibition. Furthermore, it gives a clear picture of the differences between inhibition by organophosphorus, and by carbamate, pesticides. However, as noted below in section 3.2.5, the document may misinterpret the studies of Kaplovitz *et al.* (1984) and Blick *et al.* (1989)

Although the document adequately addresses some of the difficulties in the measurement of cholinesterase and its inhibition, it would be strengthened by emphasizing even more the findings of the Workshop on Cholinesterase Methodologies (U.S. EPA, 1992), particularly the need for more and better studies on the relevance of cholinesterase inhibition (erythrocyte, plasma and brain) measurements. This includes not only the need for better and more standardized methods to compare results among laboratories but also the use of these measurements either as biomarkers of exposure or as correlates to data on clinical signs and symptoms.

As noted throughout this review, the Committee is especially concerned about species differences--the effects of which must be clearly understood in making extrapolations from animals to humans in risk assessment. In this regard, additional information could be added, such as that provided in the National Academy of Sciences (NAS) review of anticholinesterases and anticholinergics (NAS, 1982).

One other area needs to be addressed in the Forum document related to the peripheral effects of anticholinesterases. The document seems to conclude that important effects of these agents lay primarily within the central nervous system. It has been known for some time, however, that organophosphorus agents and other anticholinergics bring about immediate damage to motor endplates, an adverse effect of these agents **outside** of the central nervous system. This peripheral effect takes days-to-weeks to disappear, and may damage an appreciable number of muscle fibers (Laskowski *et al.*, 1975; Wecker and Dettbarn, 1976; Leonard and Salpeter, 1979; Dettbarn, 1984; De Bleecker *et al.* 1992).

In agonist-induced myopathy in rodents, the muscle necrosis is first noted near the neuromuscle junction. There is an increase in large-diameter vesicles in the cytoplasm beneath the junction, dissolution of Z-disks, dilatation of mitochondria and destruction of the sarcoplasmic reticulum (Leonard and Salpeter, 1979). Less than ten percent of muscle fibers may appear to be affected at the light microscope level under conditions where many more show damage under the electron microscope (Dettbarn, *ibid.*). The situation is repaired within one-to-two weeks. Damage is evident as early as 30 minutes after exposure to paraoxon. Exposures of two hours with a loss of approximately 85% of AChE activity causes severe muscle fiber necrosis.

Several lines of evidence indicate the damage is caused by inhibition of cholinesterases at the motor end plate, producing an excess of acetylcholine, which in turn leads to an increase in intracellular CA^{++} that presumably activates proteolytic enzymes leading to muscle necrosis For example; (a) Botulinum toxin type A blocks quantal release of acetylcholine and protects muscles from DFP (Diisopropyl Fluoro Phosphate) -induced lesions (Sket *et al*, 1991; Salpeter *et al.* 1979); (b) the extent of muscle damage induced by pyridostigmine is reduced by pretreatment with the calcium blocker diltiazem (Meshul, 1989); (c) the myopathy is blocked *in vitro* by removing Ca^{++} with EGTA (Ethylene Glycol-bis (beta-aminoethyl ether) N,N,N',N', Tetra acetic Acid). Several workers report the severity of the necrosis is correlated with the degree of AChE inhibition (Wecker *et al.* 1978; De Bleecker *et al.* 1991). Many of the studies were conducted with levels of anticholinesterase agents that produced major cholinergic symptoms requiring treatment with antidotes, but a recent study found necrotic fibers in low-dose poisoned rats as well (De Bleecker *et al.* 1991). Such findings suggest that peripheral "adverse" effects produced by anticholinesterase agents might be monitored by blood cholinesterase measurements. The degree to which this agonist-induced muscle necrosis in rodents is found in the human is not known.

Recently, OP poisoning cases loosely classed under the rubric of "Intermediate Syndrome" have been described and studied in the human. The first reports were from Sri Lanka where symptoms of neuromuscle damage appeared 1-4 days after poisoning with fenthion, dimethoate, monocrotophos and methamidophos (Senanayake and Karalliede, 1987) . Several other cases have been reported and studied since then (e.g. Samal and Sahu, 1990; Karademir *et al.* 1990; De Wilde *et al.* 1991, Van den Neucker *et al.* 1991) The "syndrome" is characterized by respiratory paralysis, cranial motor nerve palsies, proximal limb muscle, and neck flexor, weakness. In addition to the OPs mentioned above, a mixture of parathion and methyl parathion, diazinon, diclofenthion,

fenitrothion, dicrotophos (Bidrin) and malathion have all been associated with such symptoms. Although antidote treatments (atropine and praldoxime) have not been considered a factor by some, Benson (1992) raises the question of whether the doses used were too low. Blood cholinesterase levels have been used to monitor the time course of the 'Intermediate Syndrome;" symptoms have been reported to subside as ChE levels return to normal. The extent of damage at low levels of pesticides is not known. Those studying these poisonings usually distinguish the "Intermediate Syndrome" from organophosphate-induced delayed neuropathy (OPIDN).

3.2 Agency Positions Vis-a-vis Available Data

3.2.1 Use of Clinical Effects in Risk Assess.nent

Because it means different things to different people, the definition of "clinical effects" was first clarified by the Committee to include both overt clinical signs and symptoms in humans and behavioral changes in animals. In general, an observable "clinical" effect following a chemical exposure starts with the interaction between that chemical and a specific macromolecular "receptor" within the organism. In the case of cholinesterase-inhibiting pesticides, the specific "receptor" involved in initiating toxicity is generally agreed to be the enzyme acetylcholinesterase (AChE). Cholinergic neurons rely on AChE for the degradation and rapid termination of the synaptic transmitter (acetylcholine) in the dynamic regulation of cholinergic neurotransmission. Following extensive inhibition of AChE, acetylcholine accumulates at synaptic terminals of the central and peripheral nervous system, with subsequent over-activation or blockade of those cholinergic pathways. Through elevation of synaptic acetylcholine levels, cholinesterase-inhibiting pesticides produce typical signs of acute toxicity, including autonomic dysfunction such as excessive salivation, lacrimation, urination and defecation (SLUD signs), and evidence of neuromuscular disorder such as muscle fasciculations and motor weakness.

In general, it was the Committee's belief that such clinical effects associated with exposure to cholinesterase inhibitors could indeed be used within the context of risk assessment to establish benchmark doses and reference doses, but not without also considering other relevant toxicological information. The characteristic clinical effects produced by exposure to cholinesterase inhibitors are the toxic manifestations which typically warrant strict regulation of these pesticides. Therefore, exhibition of such typical signs associated with exposure to cholinesterase inhibitors, either in controlled

human or animal studies, should undoubtedly be useful indicators of toxicity for establishment of reference doses when other supportive toxicological data are available, and when the clinical effects are reproducible, quantifable and statistically significant, and exhibit a logical cause-effect, dose-response relationship.

A caveat to this view is that other clinical changes can occur after exposure to chemicals, including cholinesterase inhibitors, which may or may not be considered as adverse effects. In addition, as evidenced by some of the reports reviewed by the Committee, dose-related changes in such clinical effects are sometimes not evident; often the statistical estimates of treatment-related clinical effects, in particular with low dose exposures, fail to reach significance. The problems associated with using clinical signs for the establishment of reference doses for cholinesterase inhibitors, especially when the clinical endpoints cannot undoubtedly be associated with cholinesterase inhibitor exposure (e.g., a transient change in blood pressure) introduce additional uncertainty to the risk assessment process. Furthermore, there are practical problems. For example, a) the signs and symptoms associated with different cholinesterase inhibitors may not always occur in the same order; b) they depend on the rate of exposure to the toxicant and whether or not the toxicant needs to undergo metabolic activation; and c) they tend to be qualitative in nature and therefore difficult to grade as to severity (although the Committee notes that EPA is working on this problem and encourages this endeavor).

Although the sequence of events outlined above is generally adequate to explain acute toxic manifestations following exposures to cholinesterase-inhibiting pesticides, repeated exposure to these chemicals can produce dramatically different toxic sequelae. Cellular compensatory responses (e.g., modulation of the density of cholinergic receptors) can also occur in response to AChE inhibition, within hours of exposure, and these changes can modify the degree of clinical dysfunction (for a review see Costa et al., 1982). It should be stressed that changes in cholinergic receptor populations and changes in response to cholinergic agonists or antagonists (both indirect indicators of tolerance) can occur even in the absence of overt signs of toxicity (Costa *et al.*, 1982, Pope *et al.*, 1992). These compensatory responses, while thought to be prominent in the development of tolerance to the AChE inhibitor, can thus alter sensitivity to subsequent environmental or therapeutic challenges. Repeated exposures to cholinesterase-inhibiting pesticides could, therefore, fail to produce the typical overt signs of acute toxicity in animals or persons exhibiting extensive changes in neurochemistry.

In addition, the available literature suggests that all cholinesterase inhibitors do not act the same, i.e., different inhibitors may produce somewhat different spectra of toxic effects through the relative predominance of either central or peripheral involvement or through interaction with other macromolecular targets. For example, as detailed in section 3.2.3, some cholinesterase inhibitors can readily cross the blood-brain barrier while others have considerable difficulty entering the central nervous system; this differential central/peripheral involvement can dramatically affect the type and degree of "clinical" effects associated with exposure. Recent reports also suggest that some organophosphorus agents or their active metabolites (oxons) may bind directly to muscarinic receptors at concentrations significantly lower than those required to inhibit AChE (Volpe *et al.*, 1985; Bakry *et al.*, 1988; Jett *et al.*, 1991). Therefore, while the specific molecular target and the mechanism of acute toxicity for this class of compounds is well known, the ultimate expression of clinical signs following exposure to a cholinesterase inhibitor can be under complex regulation by biochemical events simultaneous or subsequent to binding to and inactivating the molecular target molecule, AChE. The conclusion is that clinical signs may be limited or even masked, in a time-dependent manner, in animals or persons severely poisoned by cholinesterase-inhibiting pesticides through the activity of alternate biochemical mechanisms. **Therefore, the sole use of clinical signs of toxicity (especially in long-term exposure studies) or perhaps more importantly, the lack of such signs for the assessment of hazard and determination of reference or benchmark doses, is not justified.** Inclusion of biochemical data regarding cholinesterase inhibition in conjunction with these signs and symptoms is considered essential for the complete hazard evaluation for these compounds.

3.2.2 ChEI in Plasma And RBC As A Biomarker of Exposure

Originally defined by their relative sensitivity to different inhibitors, the cholinesterases can be operationally divided into two types: acetylcholinesterase or "true" cholinesterase and butyrylcholinesterase or "pseudo" cholinesterase. Although AChE, the target for cholinesterase inhibitors pertinent to induction of acute toxicity, is located within the terminal regions of the central and peripheral nervous system, it is also found in tissues where no cholinergic function is apparent (e.g., erythrocyte membrane). Pseudocholinesterase (PChE) is found in the nervous system but typically in very low concentrations. PChE is also abundant in the plasma and serum, but there is no known function for PChE in any of these tissues.

In the soluble fraction of blood (i.e., either serum or plasma) in humans PChE is the overwhelmingly predominant cholinesterase, whereas rat plasma contains AChE and PChE activities in approximately equal amounts. As stated before, although there are no known functions for either PChE or AChE in the blood, inhibition of these enzymes has been historically used as a biomarker of exposure to cholinesterase inhibitors. It is generally thought that blood AChE, whether in the erythrocyte or in the plasma (in species which express that activity), responds similarly to inhibitors as does the AChE in the nervous system. In contrast, PChE may exhibit marked differences in sensitivity to some inhibitors compared to AChE. This dichotomy in response to inhibitors between the PChE and AChE has suggested to some that erythrocyte AChE activity may be a better marker for sensitivity to inhibition in "target" tissues. However, very few good correlative studies have been performed to examine the relationship between sensitivity of blood AChE, blood PChE and target AChE activities in *vivo* and therefore the relative importance of these two blood cholinesterases for prediction of target effects is unknown.

There was full agreement among the Committee members that inhibition of blood cholinesterase activity (either in plasma or erythrocytes) provides an indication of previous exposure to a cholinesterase-inhibiting pesticide, i.e., that blood cholinesterase inhibition is a biomarker of exposure. As indicated above, information regarding inhibition of the blood enzymes is often crucial supporting data for confirming exposures and corroboration of clinical signs. In addition, inhibition of ChE is regarded as a sign of a depleted enzyme reserve. Although probably not deserving to be considered an adverse effect, such depletion is itself a sign of heightened vulnerability to adverse effects from subsequent exposures. **There was strong support for the continued inclusion of blood cholinesterase data in the risk assessment process, in particular in human studies where cholinesterase data from other target tissues are unavailable.**

3.2.3 ChEI in Brain Tissue And Benchmark/Reference Doses

Although the concept itself is very attractive, there are several problems with the use of cholinesterase inhibition in animal brain tissue as a means of defining hazard and calculating benchmark or reference doses:

a) ChE inhibitors may be neurotoxic, even lethal, without significant entry into brain tissue

b) Only certain laboratory animals (rodents) are practically useful for controlled experiments

c) Inbred rodents matched for all significant factors show a marked variability in brain response to ChE inhibitors (Jimmerson *et al.*, 1989)

d) Although neuro-behavioral abnormalities may be related to CNS ChE inhibition, the correlation may be imperfect, presumably because the status of spinal cord and, in particular, peripheral ChE plays a significant role.

Toxic agents circulating in the blood have immediate access to certain regions of the nervous system that normally lack a regulatory interface ("barrier") between blood and neural tissue. These regions include the segmental spinal ganglia (containing sensory neurons), autonomic ganglia (some containing cholinergic receptors), and the circumvetricular organs of the brain (subfornical organ, area postrema, etc.). Additionally, the absence of a perineural barrier at the neuromuscular junction may increase access of blood-borne agents to this ChE-critical site. Certain ChE inhibitors are unable to traverse the blood brain and blood-nerve barriers that confront them in other regions of the nervous system, yet these agents nevertheless may produce severe neurotoxic effects, presumably through actions at peripheral sites. In these cases, measurement of CNS AChE activity would be of little or no relevance, and measurement of activity at functionally significant peripheral sites poses severe technical hurdles. **The Committee was concerned that, in general, the EPA document did not give adequate weight to the problems associated with the inhibition of peripheral nervous system cholinesterase.**

ChE inhibitors that traverse the blood-brain and blood-nerve barriers may inhibit AChE variably and with regional specificity. For example, studies with soman demonstrate that the cortex and hippocampus are more vulnerable than the striatum to systemic exposure (Jimmerson *et al.*, 1989). Studies with soman and other ChE inhibitors show variability in the degree of enzyme inhibition in a uniform group of animals and a variable correlation between the degree of enzyme inhibition and neurobehavioral sequelae or lethality. Furthermore, the data base is too small to determine whether these results are compound-specific or dose-specific, or related to other factors, such as the rate of drug administration to the animal type or the investigator undertaking the experiment.

There is presently limited understanding of the relationship between regional (brain, spinal cord) cholinesterase inhibition and neuro-behavioral sequelae. Effects on learning and memory are under study, but it is presently unknown if these will prove sensitive indicators of functional brain (e.g. hippocampus, cortex) cholinesterase perturbation. There is much more complete understanding of the peripheral somatic and autonomic targets of cholinesterase inhibition and the functional consequences for the animal (and for humans).

A major challenge in resolving the relation between brain cholinesterase inhibition and behavioral outcome is to determine the most sensitive region and the physiological functions for which it is responsible. While this alone would be a significant advance, it will provide no information on the enzyme levels at sub-regional sites (specific synapses) sensitive to cholinesterase inhibitors. Resolution of these issues and other applications to risk assessment will require the combined expertise of the neurophysiologist, morphologist, pharmacologist/toxicologist and neuro-behavioral scientist working in concert. For their results to be of use in the regulatory climate, they will need to show the relationship between agent dose, extra-cellular concentration, sub-regional cholinesterase depletion and functional change.

Although the foregoing discussion outlines the uncertainties surrounding the relationship of ChE inhibition in blood and brain tissues of animals treated with anticholinesterase agents, for certain agents there are nevertheless extant data for brain ChE inhibition in the presence or absence of corresponding data for the blood enzyme. In the risk assessment process for those anticholinesterase chemicals/metabolites known to cross the blood/brain barrier and to inhibit brain ChE, the Agency may continue to consider brain ChE data if corresponding information on blood ChE inhibition indicates the brain enzyme is a more sensitive marker of toxicity. However, the Agency should not use brain ChE inhibition data if information on the blood enzyme is unavailable; rather the Agency should take steps to obtain these data.

3.2.4 Using RBC ChEI Data for Risk Assessment

The Committee reached no simple "yes" or "no" answer on the question of using cholinesterase inhibition, by itself, for risk assessment purposes. The Committee would be very much concerned about the need to set an RfD for a chemical in which the only data available were cholinesterase inhibition measurements. Conflicting evidence exists from recent studies of neonatal and adult rats treated with common organophosphorus

pesticides. Pope and Chakraborti (1992) reported (for methyl parathion, parathion, and chlorpyrifos) that under defined conditions plasma cholinesterase inhibition may be a useful quantitative index for the degree of brain cholinesterase inhibition following exposure to organophosphorous agents. However, the degree of correlation would be inhibitor specific and could be significantly affected by factors such as the route of exposure and the lapsed time between treatment and cholinesterase inhibition measurement. A priori, the clearest window of opportunity to measure the effects in blood would be shortly after exposure, the degree of correlation falling off with time. A more complex picture would exist for agents that are metabolized to active ChE inhibitors. Additional factors affecting the correlation between blood and brain cholinesterase activity would exist for carbamates.

The correlation between plasma (or red blood cell) and brain cholinesterase activity may also be poor, for the reasons outlined above. The Jimmerson *et al.* (*ibid*) study of soman-treated rats found a poor correlation over that portion of the dose-response curve where acute toxicity occurs. They attributed the result to differences in the magnitude of change of cholinesterase in blood versus brain. They also noted that differences in the rate of enzyme inactivation may be an important variable in dictating behavioral signs of toxicity. A similar conclusion may be apparent in human subjects treated with potent nerve agents under controlled experimental conditions, as in the 1958-1975 U.S. Army program (NAS, 1982). The EPA is encouraged to review the original data from this study since they represent the largest body of information collected from human subjects treated with potent AChE inhibitors.

Although broadly applicable correlations between blood ChE and brain ChE inhibition have yet to be established, there are strong theoretical reasons why the correlation should exist for agents that traverse the blood-brain barrier. Simply stated, a direct-acting ChE inhibitor that traverses the blood-brain barrier should first inhibit plasma and RBC enzyme and, somewhat later, the brain enzyme. Provided regeneration of enzyme does not occur instantaneously, a time should exist shortly after dosing when brain enzyme inhibition is related to blood enzyme inhibition. Even if this is difficult to demonstrate empirically, it is nevertheless true that blood enzyme inhibition precedes (and therefore predicts) brain enzyme inhibition. Given the absence of any other test of proven greater reliability, measurement of plasma and red blood cell cholinesterase inhibition should be retained. Attempts should be made to standardize the conditions for these tests in order to exploit the window of opportunity when blood and brain cholinesterase inhibition are well correlated.

The Committee emphasized two additional points in its discussions. One is that when cholinesterase data are considered, one needs to use judgment in what constitutes "significance". This becomes a question of biological versus statistical differences. If the variations within groups are small and a very small decrease in cholinesterase activity is judged as statistically significant, what is the biological relevance? On the other hand, if there is a large decrease in activity but the variances are great, there would still be some concern even though the findings did not reach significance because of the biological (individual variation and/or small sample size) or technical (poor quality of the measurements) differences. A second concern is that, depending on the timing of the measurement, cholinesterase inhibition in the absence of clinical signs and symptoms may act as a portent of things to come or, as pointed out by the EPA, may show that the cholinesterase which is acting as a protective sink for the inhibitor is compromised. The Committee is concerned that, if the EPA were to base an RfD solely on clinical signs and symptoms, the possibility arises that at that dose there would be a large inhibition of cholinesterase. The Committee advises EPA against such an action until this question is resolved. We suggest that the current data sets available to the Agency be reviewed to evaluate the likelihood of such an outcome.

3.2.5 Utility of RBC Acetylcholinesterase Inhibition in Risk Assessment--Case by Case Basis

This element of the Charge postulates an assessment situation in which there is an assumption of a consistent, predictive relationship between red blood cell cholinesterase and neurotoxicity. As it is posed, the reasoning behind this question appears to be circular. If indeed one has the data on neurotoxicity to directly evaluate the dose response relationship for the chemical of interest and to determine the RfD or benchmark dose based on clinical endpoints, why go to a surrogate? However, at the Committee's meeting, the EPA clarified this to suggest that these factors - clinical effects and cholinesterase inhibition - would be used in conjunction with each other and that it was not simply a replacement of the clinical data with the erythrocyte cholinesterase inhibition data. This is, of course, the main point that was made by the Committee, i.e., analysis of all the appropriate data. **Thus, there is general agreement on the basic usefulness of the erythrocyte cholinesterase data.**

The Committee did express some concerns. The cholinesterase data must have a similar time frame to the clinical data. That is, for many of the very fast acting and readily reversible inhibitors, particularly the carbamates, the blood samples must be

obtained soon after exposure. **For organophosphorus agents, the prolonged inhibition of the erythrocyte cholinesterase beyond the period of signs and symptoms of cholinesterase inhibition needs to be kept in mind.**

The document seems to suggest that there is no direct correlation between the inhibition of erythrocyte cholinesterase and neurotoxicity. As noted above, this is not wholly true (although there is a certain window in time wherein it is correct). Part of the problem may stem from an inaccurate interpretation of the Kaplovitz *et al.* (1984) and Blick *et al.* (1989) studies cited in the Policy document. The Forum document states that these studies "lend support to the concept that blood ChEI is not predictive of neurological effects," but it is not clear how the interpretation was reached. The document may be suggesting that 40-50% inhibition is reached before there are signs of neurotoxicity, or it may fail to take into account the fact that the reversible nature of carbamate inhibition protected against the irreversible agents. Furthermore, interpretation of the cited experiments is complicated by the presence of supportive therapy given at the same time. **The Committee recommends that this section of the Forum document be rewritten for clarity.**

The Committee also recommends that EPA look carefully, both through the literature and via further experimentation, at this correlation of clinical signs and erythrocyte cholinesterase inhibition. How good are the correlations with respect to dose? With respect to time? With regard to linearity? Can the results be generalized either narrowly (some organophosphorus pesticides but not others) or broadly (e.g. organophosphorus agents vs. carbamate) or are they going to be very highly compound specific? The Committee was pleased to note that such research has already been started at EPA's Health Effects Research Laboratory in Research Triangle Park.

4. CONCLUSIONS

The positions presented in the draft *Guidance on the Use of Data on Cholinesterase Inhibition in Risk Assessment* reflect, in the main, the critical scientific data, and the Committee considers it to be a credible document. As noted in the *Detailed Findings* section of this report (section 3.2.5), addressing the use of RBC acetylcholinesterase inhibition in risk assessment, however, the *Guidance* may misinterpret the findings reported in the studies of Kaplovitz *et al.* (1984) and of Blick (1989); this section of the document should be rewritten for clarity. The Committee also recommends that the document emphasize more the findings of the Workshop on Cholinesterase Methodologies, particularly a) the need for more and better studies on the relevance of cholinesterase inhibition (erythrocyte, plasma and brain) measurements; b) the need for better methods to compare results among laboratories; and c) the use of these measurements either as biomarkers of exposure or as correlates to data on clinical signs and symptoms. The Forum document also needs to consider the peripheral effects of anticholinesterases, in addition to the focus on the effects of these agents on the central nervous system.

The Agency needs to be aware that the rodent myopathy studies and the "Intermediate Syndrome" case reports support the importance of blood cholinesterase measurements as a part of monitoring the risk of OP damage to the peripheral neuromuscle system. It should begin to look for data associated with the myopathy and the "Intermediate Syndrome." There are two major data gaps:

a) Relation to other disorders: Whether or not the human and rodent phenomena are due to the same or to different mechanisms and whether some of the "Intermediate Syndrome" cases are OPIDN waits upon future study.

b) No-Effect Levels: More dose/response studies are needed to establish quantitative relationships between organophosphate levels, extent of muscle damage, type of organophosphate, and muscle and blood levels of cholinesterases. With regard to the matter at hand, such studies are needed on rats, quantifying cholinesterase levels and extent of muscle damage for several selected organophosphates.

The Committee agrees that clinical effects (as defined in section 3.2.1 of this report) associated with exposure to cholinesterase inhibitors can be used to establish benchmark doses and reference doses, but not without also considering other relevant toxicological information. In addition, the clinical effects must be reproducible, quantifable and statistically significant, and exhibit a logical cause-effect, dose-response relationship..

Also, some clinical changes can occur after exposure to cholinesterase inhibitors, as is the case with other toxicants, which may or may not be considered as adverse effects. The problems associated with using clinical signs for the establishment of reference doses for cholinesterase inhibitors, especially when the clinical endpoints cannot undoubtedly be associated with cholinesterase inhibitor exposure (e.g., a transient change in blood pressure) introduce additional uncertainty to the risk assessment process. Given these reasons, as well as other related issues addressed in detail in this report, the Committee finds that the **sole** use of clinical signs of toxicity (especially in long-term exposure studies) or perhaps more importantly, the lack of such signs, for the assessment of hazard and determination of reference or benchmark doses may not be justified. The inclusion of biochemical data regarding cholinesterase inhibition in conjunction with these signs and symptoms is considered essential for the complete hazard evaluation for these compounds.

There was full agreement among the Committee members that blood cholinesterase inhibition is a biomarker of exposure, and that data regarding inhibition of the blood enzymes are often crucial supporting data for confirming exposures and corroborating clinical signs. We recommend that the Agency's policy continue to include the use of blood cholinesterase data in the risk assessment process, particularly in human studies where cholinesterase data from the target tissues of most concern (i.e., brain and peripheral nervous system) are unavailable.

The Committee noted several problems (detailed in the report) with the use of cholinesterase inhibition in animal brain tissue as a means of defining hazard and calculating benchmark or reference doses. Certain cholinesterase inhibitors are unable to traverse the blood-brain barrier, yet these agents may produce severe neurotoxicity, presumably through actions at peripheral sites. In these cases, measurement of CNS AChE activity would be of little or no relevance. The Committee was concerned that, in general, the EPA document did not give adequate weight to the problems associated with the inhibition of peripheral nervous system cholinesterase. A major obstacle to

resolving the relation between CNS cholinesterase inhibition and behavioral outcome is the current inability to determine the most sensitive brain regions and the physiological functions for which they are responsible. Resolution of this and other relevant issues will be required to show the relationship between agent dose, extra-cellular concentration, sub-regional cholinesterase depletion and functional change in order to support the use of brain cholinesterase inhibition data in the regulatory climate with confidence. These issues not withstanding, there are nevertheless extant data for certain agents on brain ChE inhibition in the presence or absence of corresponding data for the blood enzyme. In the risk assessment process for those anticholinesterase chemicals or metabolites known to cross the blood/brain barrier and to inhibit brain ChE, the Agency may continue to consider brain ChE data if corresponding information on blood ChE inhibition indicates the brain enzyme is a more sensitive marker of toxicity.

The Committee could not provide a simple yes or no answer to the issue of using RBC cholinesterase inhibition data by itself (i.e., in the absence of clinical symptoms) for risk assessment purposes. Extant animal studies provide conflicting evidence. Although broadly applicable correlations between blood ChE and brain ChE inhibition have yet to be established, there are strong theoretical reasons why the correlation should exist for agents that traverse the blood-brain barrier. However, even if this is difficult to demonstrate empirically, it is nevertheless true, for such agents, that blood enzyme inhibition precedes (and therefore predicts) brain enzyme inhibition. Given the absence of any other test of proven greater reliability, measurement of plasma and red blood cell cholinesterase inhibition should be retained in EPA's policy, not as the sole basis for standard setting, but in conjunction with clinical signs and symptoms. Depending on the timing of the measurement, cholinesterase inhibition in the absence of clinical signs and symptoms may predict impending adverse clinical effects. The Committee recommends that EPA evaluate the possibility that an RfD could be set based on clinical signs and symptoms that would be associated with a significant inhibition of cholinesterase occurring at a specified dose. This question could be examined with current data sets available to the Agency.

The issue of using red blood cell cholinesterase inhibition for risk assessment, as posed originally in the Charge to the Committee, was difficult to address because it appeared to be a circular argument. If there are data on neurotoxicity to evaluate directly the dose response relationship for the chemical of interest and to determine the RfD or benchmark dose based on clinical endpoints, why go to a surrogate? However, at the Committee's meeting the EPA clarified this to suggest that these factors -- clinical

effects and cholinesterase inhibition -- would be used in conjunction with each other and that it was not simply a replacement of the clinical data with the erythrocyte cholinesterase inhibition data. This is, of course, the main point that was made by the panel, i.e., analysis of all the appropriate data. Thus, there is general agreement on the basic usefulness of the erythrocyte cholinesterase data in this context.

The Committee also recommends that EPA continue research aimed at examining carefully the correlation of clinical signs and erythrocyte cholinesterase inhibition, particularly regarding the correlations with respect to dose, time, and linearity. Such studies will be very important in future decisions on the usefulness of erythrocyte cholinesterase inhibition in regulatory decision-making.

5. GLOSSARY

AChE	Acetylcholinesterase
ChE	Cholinesterase
ChEI	Cholinesterase Inhibition
CNS	Central Nervous System
DFP	Diisopropyl Fluoro Phosphate
EGTA	Ethylene Glycol-bis (beta-aminoethyl ether) N,N,N',N', Tetra acetic Acid
EPA	Environmental Protection Agency
NAS	National Academy of Science
OP	Organophosphorous
OPIDN	Organophosphate-induced Delayed Neuropathy
PChE	Pseudo Cholinesterase
RBC	Red Blood Cell
RfD	Reference Dose
SAB	Science Advisory Board
SAP	Science Advisory Panel
SLUD	Salivation, Lacrimation, Urination and Defecation

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