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Blood Esterase Determinations as Markers of Exposure

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Contents

I. Introduction

Organophosphate (OPs) and organocarbamate (OCs) esters are widely used pesticides. Their inhibition of cholinesterases (ChEs) is a major source of their toxicity. The need for detecting exposure to OPs and OCs has resulted in continuing interest in assaying for ChEs. This paper discusses assay techniques, their usefulness as indicators of exposure, and the problem of detecting adverse effects (Weiss 1990; Wilson et al. 1992a).

Even though much attention has been paid to monitoring ChEs, the only state in the USA to require ChE testing of workers in the agricultural workplace is California (State of California Department of Health, Epidemiological Studies Laboratory, AFC59-122, 1974). An investigation is required if ChE levels fall below 70% of baseline. The state of California and the

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World Health Organization (WHO) recommend removal of an individual from the workplace if red blood cell (RBC) ChE activity falls below 70% or 75% of baseline, respectively (Ames et al. 1989; Environmental Health Criteria 63 1986). Nevertheless, the lack of approved standard operating procedures in use by all clinical laboratories limits the effectiveness of the assays in establishing that meaningful exposures have occurred.

It is sobering to note that much of the research on anticholinergic chemicals has been directed at chemical warfare agents rather than concerns for the safety of food in the marketplace and workers in the agricultural workplace. Unfortunately, the differences in kinetics of OPs and OCs used in chemical warfare and agriculture limit the applicability of such research to pesticides.

II. Basic Biochemistry and Physiology

Cholinesterases such as acetylcholinesterase (AChE, EC 3.1.1.7), nonspecific cholinesterases (BChE, EC 3.1.1.8), and carboxylesterases (CaE, EC 3.1.1.1) hydrolyze esters at serine active sites. With the exception of the esterases at motor endplates and synapses of the nervous system that break down the neurotransmitter acetylcholine (ACh), their physiological functions are unknown. Another important group of enzymes are the arylesterases that include the "A-esterases" that hydrolyze paraoxon and other OPs (Aldridge and Reiner 1975; Mackness et al. 1987).

AChE occurs in two different molecular forms. "G" forms are globular, whereas "A" forms have collagenlike tails. G forms exist as monomers, dimers, or tetramers; A forms exist as tetramers, octamers, or dodecamers. Current evidence suggests that all molecular forms evolved from a common gene and contain enzymologically equivalent catalytic subunits. The three-dimensional structure of AChE from the electric organ of *Torpedo californica* has recently been established. Among the interesting features noted is that the active site, which is embedded in a "gorge" reaching halfway into the protein, and the oft-invoked "anionic site," theorized to bind the quaternary ammonium ion of ACh and similar charged structures in synthetic chemicals, appears to be a region involving 14 aromatic amino acid residues in the gorge (Sussman et al. 1991).

Properties of AChE and BChE activities are compared in Table 1. Differences include substrate preference, inhibition by excess substrate, and, in one report, divalent cation activation. The enzymes are distinguished from each other during assays by use of selective inhibitors and substrates. It is important to do so, since not all OPs and OCs inhibit both classes of enzymes to the same extent. Tissue-bound ChEs (such as RBC AChE) have longer biological half-lives than soluble enzymes (such as serum AChEs or BChEs). CaEs in the blood and other tissues bind and are inhibited by OPs,

Table 1. Properties of Mammalian Blood Cholinesterases

	AChE	BChE
Substrates	Acetyl esters	Butyryl/Proprionylesters
Selective inhibitor	BW 284c51	iso-OMPA
Excess acetylcholine	Inhibits	Ineffective
Site	RBC>>serum	Serum only
Multiple forms	Yes	Yes

but unlike the ChEs, the CaEs do not mimic enzymes in the central nervous system important to the regulation of signal transmission.

The AChEs, BChEs, and CaEs are major targets of natural and synthetic OPs and OCs. The most important acute effect of most OPs and OCs is inhibition of AChE activity in the central and peripheral nervous systems.

There are many reviews of the properties of anticholinergic organophosphorus and carbamate insecticides; two general ones are published by the World Health Organization (Environmental Health Criteria 63 1986; Environmental Health Criteria 64 1986).

The activity of ChEs at any given time after exposure to an OP or OC is dependent on several factors, including (1) inhibitor activation (conversion of sulfones to oxons by liver activity), (2) inhibitor destruction by enzymes (i.e., paraoxonases and DFPases), (3) reaction of the agent with the ChE and loss of the "leaving group," (4) spontaneous reactivation (regeneration of active enzyme by hydrolysis of bound inhibitor), and (5) aging (loss of a second group) (Environmental Health Criteria 63 1986; Environmental Health Criteria 64 1986; Aldridge and Reiner 1975). In general, OP-inhibited enzymes spontaneously reactivate more slowly than carbamates. Half-lives of reactivation of carbamylated ChEs range from several minutes to several hours. Half-lives of phosphorylated ChEs range from several hours to days (Wilson et al. 1992b). Aging of OPs is an important phenomenon; it prevents the OP-enzyme complex from spontaneously reactivating. Rates of aging range from minutes to hours and days.

Analysis of the significance of a measurement of blood ChE activity must also consider (1) the pharmacokinetics of the toxicant itself as a function of individual tissues as well as whether or not the toxicant was active or metabolized, (2) the extent of ChE inhibition as a function of time after exposure, (3) the effect of blood flow on the extent of ChE inhibition, (4) the type of ChE measured, that is, AChE or BChE, (5) the ability to correlate the extent of ChE inhibition in blood to that in other tissues, particularly regions of the brain and (6) the ability of CaEs to alter toxicant pharmacodynamics.

Table 2. Major ChE Assay Methods

Assay	Method	Reagents
Michel	pH change	Acetylcholine
pH stat	NaOH titration	Acetycholine
Radiometric	Scintillation counter	^{3}H-acetylcholine labeled acetate
Ellman et al.	Colorimetric 412 nm	Acetylthiocholine dithionitrobenzoate
Hestrin	Colorimetric 515 nm	Hydroxylamine ferric chloride

The opportunity to monitor the state of important ChEs and other esterases in the body without surgical intervention is provided by the presence of ChEs similar to those in the nervous system in particulate and serum portions of the blood. AChE forms are found in erythrocytes (RBCs), T-lymphocytes, and the serum of many mammals including humans. BChE forms are found in the serum. (AChE activity is often found in the serum of mammals and birds. Birds studied to date do not have RBC ChEs.) A major difference between human and rat serum ChEs is that rats may have 30–50% serum AChE, whereas human serum AChE levels are much lower. The properties of AChE in mammalian RBCs have been reviewed recently by Lawson and Barr (1987).

III. Methods of Cholinesterase (ChE) Measurement

A. Historical

Early methods of measuring ChE activity were reviewed by Witter (1963). One of the first methods for determining ChEs suitable for analyses of serum and tissues was the manometric technique, in which the change in pH due to hydrolysis of ACh leads to the release of carbon dioxide from the reaction buffer. The increase in acidity that accompanies the breakdown of ACh was adapted to directly determine ACh hydrolysis by examining changes in pH (Michel 1949; Table 2) or by titrating the acid produced with NaOH to keep the pH constant (Nabb and Whitfield 1967). A major use of the Michel method was the U.S. Army's program monitoring all personnel working with anticholinergic agents, whether chemical warfare agents or pesticides (personal communication, Major Dotson, Chief, Cholinesterase Quality Assurance, Fitzsimmons Army Hospital, Aurora, CO). Another technique of the period was that of Hestrin (1949), in which the reaction of ACh with hydroxylamine under alkaline conditions was followed by reaction with ferric chloride in acid solution to form a reddish-purple complex read at 515 nm. Comparisons of these methods for different animals and tissues may be

found in the literature (e.g., Hawkins and Knittle 1972). Still another assay is that of Okabe et al. (1977), in which the choline released from acetylcholine is oxidized and the H_2O_2 produced is determined with an indicator reaction at 500 nm (Abernathy et al. 1988). A recent discussion of the assay is that of Loof in the U.S. EPA Workshop Report (Wilson et al. 1992a).

B. Ellman Assay

Today, the most commonly used assays are based on a method developed by Ellman et al. (1961), in which the hydrolysis of acetylthiocholine or related compounds is measured by coupling the production of the reactive thiol group to the cleavage of the color reagent dithionitrobenzoate (DTNB). Peak absorbance for the thionitrobenzoate produced (TNB) is at 410–412 nm. The assay has been automated, miniaturized, and adapted to measuring ChE activities of almost as many tissues and species as there are investigators to study them. An example of an assay adapted to a microplate reader assay is that of Doctor et al. (1987). Automated procedures such as those used by the state of California to monitor exposure of field workers (Knaack et al. 1978) have been recently reviewed by Chin at the EPA ChE Workshop (Wilson et al. 1992a).

One problem to be aware of with the Ellman assay is the possibility of a nonlinear reaction of the thiol reagent with the reduced glutathion system of RBCs, requiring a sample blank and wait of several minutes before beginning the assay to allow the blank to become linear.

C. Radiometric Assay

A simple and relatively rapid radiometric assay is that of Johnson and Russell (1975). It relies on the differential solubility of acetate and ACh in aqueous solutions. The reaction between enzyme and the tritiated substrate is carried out in a buffered solution; when an organic solvent is added, the acetate enters the organic phase and the ACh remains in the aqueous phase where its radioactivity is quenched. One advantage of the radiometric assay is that samples may be prepared with less preliminary treatments and less dilution than with the Ellman assay; this makes the technique useful for assaying OC-treated samples. Hemolysis of RBC, dilution of samples, and indeed, virtually any processing of tissue may tend to dissociate and/or alter the activity of OC-inhibited ChEs. Drawbacks to the radiometric assay are the expense and effort needed to dispose of the radioactive wastes that are generated and the availability of scintillation counters in clinical and field laboratory settings.

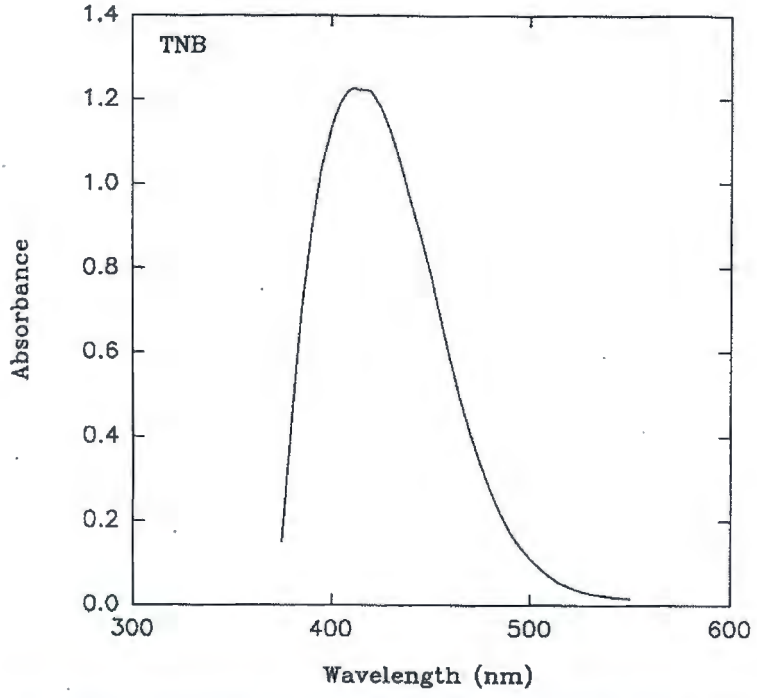

Fig. 1. Absorption spectrum of a hemolysed human RBC preparation over the major region of absorption of hemoglobin and TNB (5-thio-nitrobenzoic acid) anion reaction product.

D. Field Kits

The need for techniques that may be rapidly performed with a minimum of elaborate equipment have stimulated the development of instruments and techniques for determining the ChE activity of blood in the field. One such kit is the Test-Mate® (EQM Research, Cincinnatti, Ohio). It is based on measuring the ChE activity in a drop of whole blood using the Ellman method and an LED battery-operated device (Magnotti et al. 1987, 1988). One problem with this modification of the Ellman assay and others using intact RBCs or hemolysates is the major Soret absorption band of hemoglobin (Hb) at 410 nm, near the peak absorbance of TNB at 412 nm (Figs. 1 and 2). To avoid interference, the EQM Test-Mate® kit determinations are carried out at 470 nm, even though the absorbance of the color reagent is much reduced

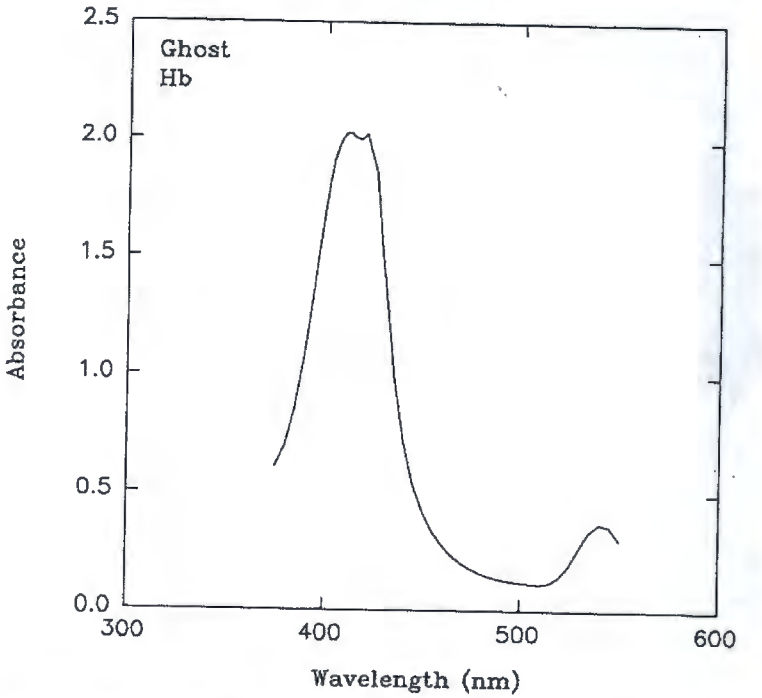

Fig. 2. Absorption spectrum of TNB over the major region of hemoglobin absorption.

at this wavelength. Ellman assay determinations of AChE activity were measured at different wavelengths using red blood cell ghosts (Table 3) and blood hemolysates (Table 4) after treatment with several concentrations of diisopropylfluorophosphate (DFP). Interference by Hb most affected the results at 410 nm and did not appear to be a factor at 470 nm. Whether the loss in sensitivity will make it difficult to detect small decreases in AChE activity under field conditions is under investigation. An alternative suggested by Loof and others is to use dithiodinicotinic acid (DTNA) that has an absorption maxima of 344 nm instead of DTNB as the color reagent (Wilson et al. 1992a).

IV. Orchard Worker Study

The reliability of ChE measurements and other tests of exposure is under investigation in a multiyear study of workers in a peach orchard previously sprayed with azinphos-methyl (Guthion)® in collaboration with the UCD Ag-

Table 3. Hb, the Ellman Assay, and DFP Treated Human RBC Ghosts

DFP (mM)	410 nm	440 nm	470 nm
0	100	100	100
1E-07	97.4	99.4	103
3E-07	89.2	89.5	96.2
1E-06	84.4	83.6	86.5
3E-06	42.7	43.6	45.7
1E-05	8.1	7.4	9.0
3E-05	0.7	0.8	3.6
1E-04	0.9	1.1	1.6

Percent untreated controls; triplicate samples. Derived from absorbance/min/ml.

riculture Health Safety Center and the California Department of Worker Health and Safety, now a part of California EPA (CEPA). Recently, the protocol was amended to include a comparison of blood ChE measurements done by a clinical laboratory, our laboratory and the Test-Mate® field kit. In addition to the ChE analyses, the overall study includes an epidemiology questionnaire, urine analyses for metabolites, and determinations of residues from t-shirts and socks. The results from our laboratory of RBC AChE activity show a progressive decrease during harvesting in each of the three years studied, especially among pickers (Table 5).

V. Standardization

One important reason for establishing standard operating procedures for ChE measurements for clinical laboratories and government bodies is to provide an even playing field for the evaluation of agricultural chemicals by health and regulatory agencies. The lack of standards favors more variable and insensitive assays, making it less likely to detect decreases in ChE levels

Table 4. Hb, the Ellman Assay, and DFP-Treated Human RBC Hemolysates

DFP (mM)	410 nm	440 nm	470 nm
0	100	100	100
1E-07	99.9	104	102
3E-07	75.3	89.1	92.7
1E-06	86.9	92.4	88.5
3E-06	60.1	52.1	48.1
1E-05	24.6	13.5	10.5

Percent untreated controls; triplicate samples.

Table 5. ChE Activity of Peach Workers in a Guthion-Treated Orchard

Year 1	7/21/89	7/31/89	9/8/89
CDFA	—	—	—
Picker	12.1 ± 0.2 (19)	11.6 ± 0.2 (18)	9.9 ± 0.5 (12)a
Sorter	12.7 ± 0.2 (12)	13.5 ± 0.5 (10)b	13.0 ± 0.3 (10)b
Year 2	**8/16/90**	**9/4/90**	
CDFA	10.7 ± 0.8 (4)	12.1 ± 0.5 (2)	
Picker	10.6 ± 0.4 (17)	9.1 ± 0.4 (10)a	
Sorter	11.9 ± 0.2 (13)b	11.8 ± 0.5 (7)b	
Year 3	**8/12/91**	**8/26/91**	**9/5/91**
CDFA	—	12.2 ± 0.1 (3)	11.8 ± 0.1 (3)a
Picker	9.3 ± 0.2 (21)	9.4 ± 0.3 (21)	7.8 ± 0.4 (15)a
Sorter	9.8 ± 0.3 (9)	11.3 ± 0.7 (7)b	10.4 ± 0.5 (2)b

Means ± Standard deviations, umoles/min/ml RBC; statistically different from (a) initial value and (b) pickers.

that might signal regulatory actions. Recently, the U.S. EPA held a workshop on cholinesterase methodologies to assess the quality of the ChE database on file used to set reference dose (RfD)* values in food products for OPs and OCs. The findings of an EPA workgroup documented (1) lack of standard operating procedures for assays from laboratory to laboratory and lack of enzyme activity standards, (2) lack of a common way of reporting the activities, and (3) biochemically inappropriate storage and assay conditions, especially with regard to OCs. Concerns of the committee included establishing reliable baseline data, the effect of transportation and storage, avoiding Hb interference, and the extent of spontaneous reactivation during processing. The panel recommended that an interim protocol be tested in the laboratories of several of the panel members, followed by an annual "round-robin" test in which samples with known activities are sent to the clinical laboratories. A similar test of the Ellman assay has recently been conducted (Harlin and Ross 1990). Once standardized, such a test could be readily modified for the monitoring of the exposure of workers to ChE-inhibiting chemicals and used as a standard to compare to other tests.

*Reference dose (RfD) represents an estimate of a daily exposure in mg/kg/d to the general public, including sensitive subgroups that are likely to be without an appreciable risk of deleterious effects during a lifetime exposure (chronic RfD), or a limited time interval (subchronic RfD).

Table 6. Reactivation of Beagle RBC AChE After Paraoxon in vitro

Sample	No Oxime	2-PAM
Control	81.6	80.6
1 uM PO	36.3	79.3
100 uM PO	3.3	99.8

nmoles/min/mL packed cells; means of triplicate samples. PO treatments for 30 min on ice. Assays at 25°.

VI. Oxime Reactivation

OPs (and OCs) are two of the few classes of agricultural chemicals for which there are clear-cut specific treatments and antidotes. Chemicals such as atropine reduce the effectiveness of the excess ACh brought about by inhibition of the ChEs and reactivators such as pralidoxime (2-PAM) and toxigonin. Oxime reactivation of ChEs inhibited by OPs may be used as a qualitative estimate of exposure, provided that aging (loss of an alkyl group) of the OP-ChE complex has not occurred. Most methods of establishing exposure to agricultural chemicals require baseline data on the individuals concerned, or access to reliable tables of normal values (Lepage et al. 1985; Sidell and Kaminskis 1975; Sanz et al. 1991; Trundle and Marcial 1988). The ability of oximes such as 2-pralidoxime (2-PAM) to reactivate OP-ChE complexes provides an opportunity to study exposure with a single sampling (Wilson et al. 1992b). A sample suspected of having been exposed to an OP is split; one aliquot is examined for AChE activity without treatment with an oxime, the other is treated with the oxime. Prior exposure to an OP is indicated if the ChE activity increases after oxime treatment. Such a test of ChE activity of RBC, plasma, or tissue ChEs could be useful in monitoring possible exposure of humans and wildlife to OPs for periods up to approximately 72 hr, depending on the OP and dose [see Wilson et al. (1992b) for a recent review]. An example of a reactivation experiment is shown in Table 6, in which blood from a beagle was exposed to paraoxon. The blood was drawn, the RBCs separated from the plasma and treated with paraoxon (PO) in an ice bath, lysed by osmotic shock, and the ghosts assayed for AChE activity with and without 2-PAM treatment (0.1 mM). Under these test conditions, 2-PAM treatment brought about virtually complete recovery of AChE activity. An example of blood ChE activities after an animal was treated with an OP is shown in Table 7. A rabbit was dermally treated for 7.5 hr with parathion, the blood sampled at the times indicated, and the RBC AChE activity determined with and without 2-PAM (Wilson et al. 1992). Reactivation was statistically detectable under these controlled conditions for several days after exposure and at levels of inhibition less than 10%. In general,

Table 7. Reactivation of Rabbit RBC AChE After Parathion in vivo

Hours	Untreated	Percent	Treated	Percent
0	2.25	0	2.36	4.8
1	1.90	15.5	2.19	15.1a
4	1.34	40.3	2.06	51.7a
8	1.01	55.1	2.00	98.3a
12	0.82	63.7	1.73	111.7a
24	1.14	49.2	1.72	50.2a
36	1.30	42.3	1.74	33.9a
48	1.46	34.9	1.81	23.5a
60	1.69	24.8	1.88	11.4a
72	1.72	23.3	1.83	6.2a
84	1.83	18.4	1.95	6.2b
108	1.98	12.0	2.07	4.7
132	2.10	6.5	—	—
360	2.06	8.2	2.13	3.1

umoles/min/mL; a: significant $P<0.01$; b: $P<0.05$. 0.1 mg/kg parathion applied dermally and removed after 7.5 hr; untreated is activity before 2-PAM treatment, followed by percent inhibition compared to 0 hour. Treated is activity after 2-PAM, followed by the percent increase in activity compared to the untreated sample at the same time point.

OP-AChE complexes should be reactivatable for several hours (nerve gases) to several days (pesticides) after treatment, depending on the rates of aging of the OP and destruction of the complex. There was little evidence of reactivation in the peach orchard/azinphos-methyl study, suggesting that the decrease in AChE activity noted was due to aged enzyme.

VII. Other Methods to Assess Exposure

A. OPIDN and Neuropathy Target Esterase

In addition to ChEs, other esterases such as neuropathy target esterase (NTE) are useful in assessing the dangers of OPs. High levels of inhibition of NTE by OPs have been associated with organophosphate-induced delayed neurotoxicity (OPIDN, Johnson 1990), and several OPs used in agriculture have been shown to cause OPIDN in experimental animals and man [reviewed in Environmental Health Criteria 63 (1986)]. Agricultural OPs recently reported to cause long-term neural damage in humans include chlorpyrifos, isofenphos, and methamidophos (Capodicasa et al. 1991; Catz et al. 1988; Samal and Sahu 1990; Senanayake and Karalliede 1987). The presence of NTE in lymphocytes affords an opportunity to monitor exposure to potential neuropathic chemicals (Johnson 1990). However, the assay currently in use requires a larger sample than for AChE alone, and the correlation of de-

creases in lymphocyte NTE activity to the risk of OPIDN is not as quantitatively established as with brain, spinal cord, and axon NTE activity. Recent progress in purifying the enzyme and establishing its substrate specificities may lead to an assay suitable for field studies (Ruffer-Turner et al. 1992; Mackay et al. 1992; Thomas et al. 1990).

B. Urinary Metabolites

The nearest "competitor" to monitoring ChE levels in blood at present is probably the determination of urinary metabolites (Weisskopf et al. 1988). The techniques are noninvasive, sensitive, and readily standardized. However, the rapid disappearance of OP and OC metabolites from the blood makes such techniques more useful for the detection of acute exposures than for revealing exposures that occurred in the past. Inhibition of RBC AChE (so long as aging has occurred) permits study of past exposure, providing allowance is made for the appearance of new RBCs and their normal level of enzyme at approximately 1%/d. One difference between studies of ChE levels and urinary metabolites is that the determination of ChE levels does not require prior knowledge of what chemicals may have been involved in the exposure.

VIII. Future Assays

The widespread use of OPs and OCs as agricultural chemicals creates a need for sensitive methods to detect exposure to them and assess their hazard to humans and wildlife. Antibody tests for the presence of an agent, its metabolites, and protein complexes may provide sensitive ways of detecting and quantifying exposures.

IX. Is a Decrease in ChE Activity an Adverse Effect?

Recently, much discussion was engendered by the proposal that a statistically significant decrease in ChE activity would define the adverse effect level of an anti-ChE chemical so far as risk assessment procedures were concerned (Weiss 1990). The panel reviewing the proposal agreed that decreases in ChE were reliable markers of exposure, but rejected the idea that small decreases in AChE indicated, in and of themselves, that the health of the organism (human, domestic, wild) had been impaired. Problems raised included the fact that ChE enzymes may be present in physiological excess in the nervous system, and that little data exist correlating small decreases (i.e., 5–10%) in ChE activities with alterations in the behavior, physiology, reproduction, or other important features of the life histories of animals exposed to anti-ChE chemicals. Risk assessment procedures require accurate

inputs of data to deliver meaningful results. For this reason, the deceptively simple matter of accurately determining exposure to agricultural chemicals such as the OPs and OCs is of fundamental importance to the establishment of safe levels of chemicals in the marketplace, farm, and environment, and we look forward to much research activity and public and private discussions in this area.

Summary

The bases of using blood enzyme activity measurements [e.g. AChE, nonspecific cholinesterase (BChE), carboxylesterase] as markers of organophosphate ester (OP) exposure are inhibition of activity by the binding of OPs to serine active sites in the enzymes, and the accessibility of the enzymes in RBCs and serum. The methods used to determine esterases in the blood of humans, experimental animals, and wildlife are outlined with emphasis on the acetylcholinesterase (AChE) of the red blood cell. Adaptations of an acetylthiocholine ester assay of Ellman et al. (1961) are common, but other colorimetric procedures, radiometric assays, and pH methods are also in use. Optimized, standardized methods are needed to assess exposures and provide a solid basis for risk assessment analyses. Useful adjuncts to ChE measurements are oxime reactivation tests and assay of neuropathy target esterase, an enzyme associated with organophosphate-induced delayed neuropathy. Determination of urinary metabolites compliments, but does not substitute for, the information obtained from blood ChE studies. Future assays are likely to involve antibodies to OP-protein complexes. Improvements in techniques permit the detection of small decreases in ChE activities. Whether or not such small decreases in ChE activities can, by themselves, constitute an adverse effect for input into risk assessment analyses is a controversial matter.

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