Human & Experimental Toxicology

http://het.sagepub.com

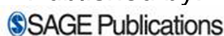
Biosensor assay of neuropathy target esterase in whole blood as a new approach to OPIDN risk assessment: review of progress

Galina F Makhaeva, Vladimir V Malygin, Nadezhda N Strakhova, Larisa V Sigolaeva, Lidia G Sokolovskaya, Arkady V Eremenko, Ilya N Kurochkin and Rudy J Richardson

Hum Exp Toxicol 2007; 26; 273
DOI: 10.1177/0960327106070463

The online version of this article can be found at:
http://het.sagepub.com/cgi/content/abstract/26/4/273

Published by:



http://www.sagepublications.com

Additional services and information for *Human & Experimental Toxicology* can be found at:

Email Alerts: http://het.sagepub.com/cgi/alerts

Subscriptions: http://het.sagepub.com/subscriptions

Reprints: http://www.sagepub.com/journalsReprints.nav

Permissions: http://www.sagepub.com/journalsPermissions.nav

Downloaded from http://het.sagepub.com at UNIV OF MICHIGAN on July 5, 2007
© 2007 SAGE Publications. All rights reserved. Not for commercial use or unauthorized distribution.

Human & Experimental Toxicology (2007) **26**: 273–282
http://het.sagepub.com

Biosensor assay of neuropathy target esterase in whole blood as a new approach to OPIDN risk assessment: review of progress

Galina F Makhaeva[1], Vladimir V Malygin[1], Nadezhda N Strakhova[1], Larisa V Sigolaeva[2], Lidia G Sokolovskaya[2], Arkady V Eremenko[2], Ilya N Kurochkin[2] and Rudy J Richardson*[,3]

[1]Institute of Physiologically Active Compounds, Russian Academy of Sciences, Chernogolovka, Moscow Region 142432, Russia;
[2]Faculty of Chemistry, M.V. Lomonosov Moscow State University, Moscow 119992, Russia;
[3]Toxicology Research Training Program, Department of Environmental Health Sciences, School of Public Health, The University of Michigan, Ann Arbor, MI 48109, USA

Organophosphates (OPs) that inhibit neuropathy target esterase (NTE) with subsequent ageing can produce OP-induced delayed neuropathy (OPIDN). NTE inhibition in lymphocytes can be used as a biomarker of exposure to neuropathic OPs. An electrochemical method was developed to assay NTE in whole blood. The high sensitivity of the tyrosinase carbon-paste biosensors for the phenol produced by hydrolysis of the substrate, phenyl valerate, allowed NTE activity to be measured in diluted samples of whole blood, which cannot be done using the standard colorimetric assay. The biosensor was used to establish correlations of NTE inhibitions in blood with that in lymphocytes and brain after dosing hens with a neuropathic OP. The results of further studies demonstrated that whole blood NTE is a reliable biomarker of neuropathic OPs for up to 96 hours after exposure. These validation results suggest that the biosensor NTE assay for whole blood could be developed to measure human exposure to neuropathic OPs as a predictor of OPIDN. The small blood volume required (100 μL), simplicity of sample preparation and rapid analysis times indicate that the biosensor should be useful in biomonitoring and epidemiological studies. The present paper is an overview of our previous and ongoing work in this area. *Human & Experimental Toxicology* (2007) **26,** *273–282*

Key words: OPIDN; neuropathy target esterase (NTE); biomarker; blood; organophosphates (OPs); tyrosinase biosensors

Introduction

Organophosphate-induced delayed neurotoxicity (OPIDN) is a central-peripheral distal axonopathy that occurs ~8–21 days after acute exposure to neuropathic OPs. Initial flaccid paralysis and sensory loss in distal extremities converts to spastic paralysis months to years after disease onset.[1,2] Axonopathy does not depend upon acetylcholinesterase (AChE) inhibition/ageing, and can be induced by OPs with low acute toxicity.[3,4] Most of the ~50 000 cases of OPIDN occurred between 1930 and 1980, and the current incidence is extremely low.[2,5] However, OPIDN is still of great concern, because humans are highly susceptible and the disease cannot be stopped once it is initiated.[6,7] These properties of OPIDN raise the spectre of neuropathic OP compounds being used as agents for chemical terrorism. Defence against such agents requires detection that distinguishes them from classical OP nerve agents. Measurement of neuropathy target esterase (NTE) inhibition in blood provides such a method.

OPIDN is initiated by the concerted organophosphorylation and ageing of NTE.[3,8–12] Chickens are more susceptible to OPIDN than rats, and adults are more sensitive than young.[13] Thus, the experimental animal model of choice for studies of OPIDN is the adult hen.[14,15] Brain inhibition/ageing of >70% of the NTE activity predicts that OPIDN will develop in hens. It is likely that a similar threshold exists for humans.[16]

*Correspondence: Rudy J Richardson, Toxicology Program, The University of Michigan, 1420 Washington Heights, Ann Arbor, MI 48109-2029, USA
E-mail: rjrich@umich.edu

Received 31 October 2005; revised 20 February 2006; accepted 16 March 2006

Downloaded from http://het.sagepub.com at UNIV OF MICHIGAN on July 5, 2007
© 2007 SAGE Publications. All rights reserved. Not for commercial use or unauthorized distribution.

© 2007 SAGE Publications 10.1177/0960327106070463

Because of the solid relationship between NTE inhibition/ageing and OPIDN, this enzyme is an ideal predictive biomarker.[3,12,17] NTE has been found in circulating lymphocytes and platelets,[18–21] and lymphocyte NTE has been used or proposed for use as a biomarker of animal and human exposure to neuropathic OP compounds.[10,21–25] In addition, lymphocyte NTE inhibition has been suggested as a predictor of OPIDN or an adjunct for its early diagnosis.[7,21,26–28]

Although NTE in lymphocytes should be an ideal marker, it takes time, resources and relatively high sample volumes to separate lymphocytes from whole blood. Therefore, there would be an advantage to being able to detect the lymphocyte (and platelet) NTE directly in whole blood.[29] The standard NTE assay is based on the colorimetric determination of phenol released by hydrolysis of the substrate, phenyl valerate.[30,31] Because of interfering absorbances, the colorimetric assay as typically conducted cannot be used to assay NTE in whole blood.[29]

The problems inherent in a colorimetric NTE assay could be eliminated by using an amperometric technique to detect phenol produced by the NTE hydrolysis of phenyl valerate. Such an approach was developed using tyrosinase-based biosensors. These involve the enzymatic oxidation of phenol via catechol into *o*-quinone, a reaction that consumes molecular oxygen (Figure 1).[32]

The concentration of phenol can be monitored by oxygen consumption when an oxygen electrode is used as an electrochemical transducer (Figure 1, I).[33,34] Electroreduction of quinone to catechol directly on the graphite electrode can also be used as a detection reaction for the quantification of phenol.[35–37] This regenerating process amplifies the electrode response to achieve more sensitive phenol detection than is possible by measurement of oxygen consumption (Figure 1, II).[29,38] Regeneration via a mediator can give a more enhanced sensitivity to phenol compared to the direct electroreduction of *o*-quinone (Figure 1, III).

In this paper, we present an overview of our previous and current works on development of phenolic biosensors for NTE assay with emphasis on 1-methoxyphenazine methosulfate (MPMS)-modified tyrosinase carbon paste electrode; validation of the new biosensor methods of NTE analysis; demonstration of the utility of the new biosensor method for assaying NTE activity in whole blood; and use of these measurements as a biomarker of exposure to neuropathic OPs.

Methods

Chemicals

Mushroom tyrosinase (monophenol monooxidase, EC 1.14.18.1), activity 3800 U/mg for L-tyrosine, graphite powder, phenol, MPMS, paraoxon (*O*,*O*-diethyl-4-nitrophenyl phosphate), 4-aminoantipyrine and potassium ferricyanide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phenyl valerate was from Oryza (Chelmsford, USA). *N*,*N*′-Di-2-propylphosphorodiamido fluoridate (mipafox), *O*,*O*-di-1-propyl *O*-2,2-dichlorovinylphosphate (PrDChVP) were synthesized and characterized in the Institute of Physiologically Active Compounds, Russian Academy of Sciences (RAS) (Russia). The purity of all substances was >99% (by spectral, chromatographic and elemental analysis data). The Coomassie protein kit was from Sigma Chemical Co. (USA). All other chemicals were of analytical grade and used without further purification. Aqueous solutions were prepared using deionized water.

Preparation of tyrosinase electrode and assembly of biosensor

The tyrosinase electrode was prepared as described below.[39] Graphite/paraffin oil (3.5:1, w/w) was mixed thoroughly and packed firmly into a plastic

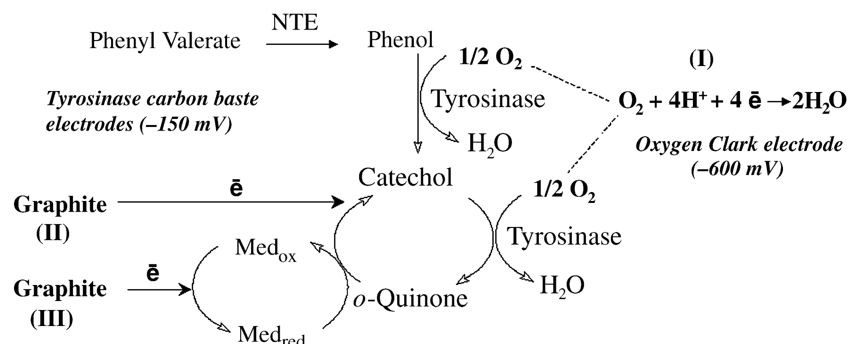


Figure 1 Principle of amperometric phenol detection and NTE analysis by different types of tyrosinase biosensors: I, Clark-type oxygen electrode; II, tyrosinase carbon paste electrode; III, mediator-modified tyrosinase carbon-paste electrode. Med_{ox}, an oxidized mediator; Med_{red}, a reduced mediator.

Downloaded from http://het.sagepub.com at UNIV OF MICHIGAN on July 5, 2007
© 2007 SAGE Publications. All rights reserved. Not for commercial use or unauthorized distribution.

holder (2 mm diameter). Electrical contact was established by a 0.2-mm copper wire inserted from the rear. The surface of the electrode was covered by the composite of tyrosinase, graphite, MPMS and paraffin oil (2:5.6:1:2, by weight), and then the surface was polished. The electrodes were stored dry at 4°C until use.

Electrochemical measurements
All measurements were performed with an IPC-2000 potentiostat (Institute of Physical Chemistry, RAS). The amperometric current response was recorded at −150 mV versus the Ag/AgCl reference electrode in a flow-through electrochemical cell. Operation of the device and processing of electrochemical measurements were done using software developed in the laboratory.

Studies in vitro
Hen brain NTE assay A lyophilized hen brain membrane fraction consisting of combined mitochondrial/synaptosomal and microsomal pellets (P_2+P_3)[40] preinhibited with paraoxon (40 μM at 25°C for 45 min) was used as a source of NTE. It was prepared as described[41,42] and stored in sealed ampoules. Before use, ampoule contents were suspended with a Potter homogenizer in 5 mL of working buffer (50 mM Tris–HCl, 0.2 mM EDTA, pH 8.0 at 25°C). NTE activity was determined colorimetrically according to the differential inhibition method of Johnson[30] as modified by Kayyali and co-workers[31] at 492 nm using a Benchmark Plus Microplate Reader (Bio-Rad, Hercules, CA, USA). Each measurement was made in triplicate. Protein was determined by the microbiuret method with bovine serum albumin as a reference standard.[43]

Whole blood NTE assay Whole hen blood stabilized by citrate/EDTA was diluted 10-fold with working buffer and homogenized in a Potter glass/glass homogenizer before NTE assay. The differential inhibition method of Johnson[30] with an electrochemical endpoint was used as described previously.[44] Each measurement was made in triplicate. Protein was determined using a Coomassie protein assay kit from Sigma (USA) with bovine serum albumin as a reference standard.

NTE I_{50} determinations The I_{50} (the concentration that inhibits 50% of the enzyme activity under defined preincubation conditions) for inhibitors against NTE was measured by preincubating a sample of enzyme with 10–12 different concentrations of the OP compound for 20 min at 37°C in working buffer. Residual NTE activity was then determined either using the colorimetric (hen brain NTE) or the amperometric (blood NTE, tyrosinase carbon-paste electrode) method.[29,44] Each measurement was made in triplicate (colorimetry) or in duplicate (amperometry). I_{50} values were calculated using Origin 6.1 software (Northampton, MA, USA). Every value represents the mean ± SEM from three independent experiments.

Experimental animal study
Animals Adult white Leghorn hens (18 months of age, 1.5–2.0 kg) were from Noginsk poultry farm (Noginsk, Russia). Hens were used under conditions that prevented them from experiencing unnecessary pain and discomfort in accordance with guidelines from the Committee for the Control and Supervision of Experiments on Animals of Russian Ministry of Education and Russian Ministry of Health as well as international recommendations (Ethical codex) for carrying out experiments on animals.[45] Hens were kept three to a stainless steel cage with food and tap water *ad libitum*. Hens were kept in a room with a 12-hour light cycle and controlled temperature (20–23°C).

Dose-dependence of blood, lymphocyte and brain NTE inhibition 24 hours after PrDChVP administration The experimental details were described previously.[44]

Dose-dependence of blood and brain NTE inhibition 4 hours after PrDChVP administration PrDChVP in acetone/water 1/1 (v/v) solvent was injected im (1.0 mL/kg) into hens at doses of 0.32, 0.40, 0.56 and 1.0 mg/kg, to groups of three hens per dose to assess NTE inhibition in brain and whole blood 4 hours after administration. All hens were pretreated with atropine sulphate in water, 20 mg/kg im (1.0 mL/kg), 20 min before PrDChVP injection. Control animals received atropine sulphate only. Four hours after PrDChVP administration, hens were decapitated under CO_2-induced anaesthesia for collection of tissue samples. Blood from each hen was collected immediately in glass vials containing a solution of 3.8% (w/v) sodium citrate and D-glucose (at a ratio of 0.20 mL anticoagulant per millilitre of blood), frozen in liquid nitrogen and stored at −20°C before NTE assay. Brains were rapidly removed, frozen in liquid nitrogen, weighed and stored at −20°C before NTE assay.

Time-dependence of blood and brain NTE inhibition PrDChVP in acetone/water 1/1 (v/v) was injected im (1.0 mL/kg) into hens at the maximum dose used in dosing Experiment I above: 1 mg/kg to groups of three hens per time to assess

Downloaded from http://het.sagepub.com at UNIV OF MICHIGAN on July 5, 2007
© 2007 SAGE Publications. All rights reserved. Not for commercial use or unauthorized distribution.

NTE inhibition in brain and whole blood 4, 24, 48, 72 and 96 hours after administration. All hens were pretreated with atropine sulphate in water, 20 mg/kg im (1.0 mL/kg), 20 min before PrDChVP injection. Control animals received atropine sulphate only. At 4, 24, 48, 72 and 96 hours after PrDChVP administration hens were decapitated, and blood and brains from each hen were collected and stored as described above for measurement of NTE activity in dosing experiments.

Brain NTE assay After being allowed to thaw at room temperature, each brain was homogenized at 4°C in 5 volumes of working buffer with a Potter homogenizer, and centrifuged for 15 min at 9000 × g at 4°C. The brain 9S supernatant was used for NTE assay[46] with substrate (phenyl valerate) using the differential colorimetric method of Johnson[30] in a microassay version[31] as described previously.[44] The measurements were carried out at 492 nm using a Benchmark Plus Microplate Reader (Bio-Rad, USA). Each measurement was made in triplicate. Protein was determined by the microbiuret method[43] with bovine serum albumin as a reference standard.

Whole blood NTE assay Frozen blood samples were allowed to thaw to room temperature, diluted 10-fold with working buffer and homogenized in a Potter homogenizer before the amperometric NTE assay. The differential inhibition method of Johnson[30] with the electrochemical endpoint was used as described before,[44] using phenyl valerate as the substrate. Prior to phenol measurements, samples obtained after stopping the enzymatic reaction with SDS were further diluted 20–50-fold in 0.1 M NaCl + 0.05 M sodium phosphate buffer, pH 7.0. Enzymatically released phenol was measured amperometrically after an injection of the diluted final reaction mixture into a flow of 0.1 M NaCl + 0.05 M sodium phosphate buffer, pH 7.0 via an injector with a 50-μL sample loop (Valve V-7, Pharmacia, Sweden). Flow rate was 0.25 mL/min. Each measurement was made in triplicate. Protein was determined using a Coomassie protein assay kit from Sigma Chemical Co. (USA) with bovine serum albumin as a reference standard.

Statistical analysis

In vitro studies All values are expressed as the mean ± SEM ($n = 3$ separate experiments). Independent two-tailed t-tests were used to determine the significance of differences between I_{50} values for a given inhibitor and tissue measured by amperometry versus colorimetry or by amperometry on hen versus human blood. The level of significance was set at $P < 0.05$. These tests were carried out using GraphPad Prism version 3.02 for Windows, GraphPad Software (San Diego, CA).

In vivo studies NTE activity in brain and whole blood from hens treated with atropine and PrDChVP was compared to activity in corresponding tissues from animals treated with atropine only (control) to calculate % inhibition relative to control. The results were analysed with one-way ANOVA using GraphPad Prism 3.0 software (GraphPad Software, Inc. San Diego, CA, USA). The correlation of NTE inhibition between brain and blood (dosing Experiment I) was examined by plotting correlation curves and calculating associated correlation coefficients (r) and P-values, using Origin 6.1 software (OriginLab Corp., Northampton, MA, USA).

Results

Influence of whole blood dilution on measured NTE activity

Using a MPMS-modified tyrosinase carbon-paste biosensor,[39] the dependence of an apparent NTE activity on amount of blood in the analysed sample (influence of level of dilution of the homogenized blood) was studied. The results presented in Figure 2 demonstrate a linear dependence of the apparent NTE activity on blood concentration when the extent of blood dilution changed from 1:275 to 1:2200.

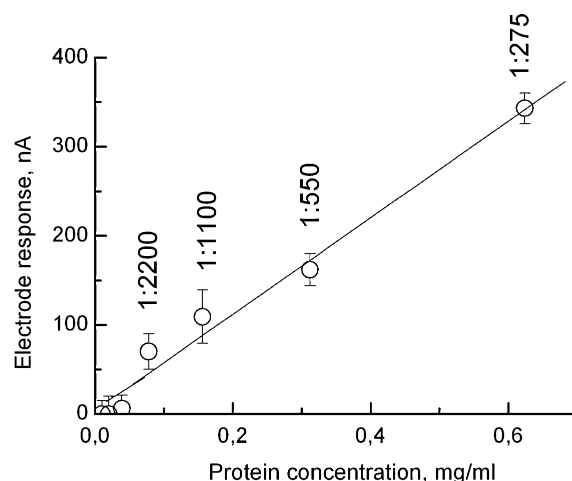


Figure 2 Dependence of the apparent NTE activity in human blood (measured as the MPMS-modified tyrosinase carbon-paste electrode response, nA) on final blood sample dilution. Conditions: 0.05 M phosphate buffer solution with 0.1 M NaCl; pH 7.0; applied working potential −150 mV versus Ag/AgCl; volume of the analysed blood in each dilution, 20 μL; incubation time, 30 min; blood NTE specific activity, 0.25 nmol phenol produced/(min × mg protein).

Downloaded from http://het.sagepub.com at UNIV OF MICHIGAN on July 5, 2007
© 2007 SAGE Publications. All rights reserved. Not for commercial use or unauthorized distribution.

Mipafox and PrDChVP I_{50} values against NTE from different sources determined by colorimetry and amperometry

To validate the biosensor measurements, I_{50} values of standard neurotoxicants mipafox and PrDChVP to hen brain NTE as well as hen and human lymphocytes were determined using the traditional colorimetric method and the tyrosinase carbon paste electrode methods.[29,44,47] Biosensor methods were used to determine I_{50} values of mipafox and PrDChVP against hen and human whole blood NTE; I_{50} values for hen blood were determined both with MPMS-modified and unmodified tyrosinase paste electrodes. The results, along with literature data on hen brain and hen and human lymphocyte NTE inhibition, are presented in Table 1.

Studies in vivo

Dose-dependence of blood, lymphocyte and brain NTE inhibition 24 h after PrDChVP administration Inhibition of NTE in hen brain and lymphocytes (Experiment I) as well in brain and blood (Experiment II) was studied 24 hours after injecting hens with increasing doses of the neuropathic OP, PrDChVP. NTE inhibition was measured in brain and lymphocytes using the colorimetric assay and in whole blood using the amperometric biosensor assay.[44] Dose-dependent inhibition of NTE was found in both experiments, as shown in Figure 3.

Dose-dependence of blood and brain NTE inhibition 4 hours after PrDChVP administration Inhibition of NTE in hen brain and whole blood was studied 4 hours after injecting hens with increasing doses of PrDChVP. NTE inhibition was measured in brain by the colorimetric assay and in whole blood using the amperometric biosensor assay. Figure 4 shows that brain and blood NTE were inhibited in a dose-responsive manner 4 hours after dosing.

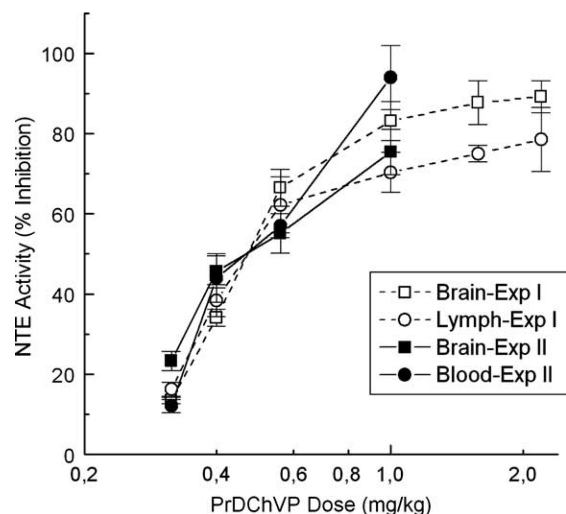


Figure 3 Dose-related NTE inhibition in brain, lymphocytes and whole blood of hens 24 hours after injection of the neuropathic OP, PrDChVP. Results are % control values for each tissue expressed as mean ± SEM, $n = 3$. Open square, dashed line (– – □ – – Brain-Exp I), brain NTE, dosing experiment I (NTE assayed colorimetrically in brain and lymphocytes; both tissues assayed fresh). Open circle, dashed line (– – ○ – Lymph-Exp I), lymphocyte NTE, dosing experiment I. Closed square, solid line (—■— Brain-Exp II), brain NTE, dosing experiment II (NTE assayed colorimetrically in brain and amperometrically in blood; both tissues assayed after freezing and thawing). Closed circle, solid line (—●— Blood-Exp II), blood NTE, dosing experiment II. Control NTE activities, nmol/(min × mg protein), mean ± SEM, $n = 3$: Dosing experiment I, brain = 30.9 ± 2.8, lymphocyte = 9.0 ± 1.4; Dosing experiment II, brain = 16.8 ± 0.88, whole blood = 0.107 ± 0.013. Copyright 2003,[44] reproduced in slightly altered form by permission of Taylor & Francis Group, LLC., http://www.taylorandfrancis.com.

Table 1 Mipafox and PrDChVP I_{50} for NTE from different sources determined by amperometry and colorimetry

Source of NTE	*Method*		
	Amperometry[b]	*Colorimetry*[b]	*Colorimetry (literature data)*
Mipafox I_{50} (μM)[a]			
Hen brain	4.32 ± 0.28[29]	4.20 ± 0.54[29]	7.9[19]; 7[48]; 3.8[41]; 6.6–8.1[49c]; 7.3[50]; 3.1[51]
Hen lymphocytes	6.94 ± 0.62[44]	6.02 ± 0.71[44]	11.2[19];12.6[21]
Hen whole blood	4.22 ± 0.12[47] 4.17 ± 0.22[e]	ND[d]	ND[d]
Human lymphocytes	8.38 ± 0.88[29]	7.58 ± 0.79[29]	10[18]; 9.6[21]
Human whole blood	6.27 ± 0.43[29]	ND[d]	ND[d]
PrDChVP I_{50} (μM)[a]			
Hen brain	0.039 ± 0.008[44]	0.042 ± 0.002[44]	0.052[48]
Hen whole blood	0.066 ± 0.003[44] 0.058 ± 0.005[e]	ND[d]	ND[d]
Human whole blood	0.070 ± 0.014[44]	ND[d]	ND[d]

[a]All I_{50} values based on using a 20-min preincubation with mipafox or PrDChVP at 37°C, pH 8.0. Reference numbers are shown for values taken from our previous studies or other literature.
[b]Each value represents the mean ± SEM for at least three independent experiments.
[c]Range of values for NTE solubilized under various conditions.
[d]ND, no data, because colorimetric determination of NTE cannot be carried out in whole blood.
[e]Determined with MPMS-modified electrode.

Downloaded from http://het.sagepub.com at UNIV OF MICHIGAN on July 5, 2007
© 2007 SAGE Publications. All rights reserved. Not for commercial use or unauthorized distribution.

Time-dependence of blood and brain NTE inhibition Inhibition of NTE in hen brain and whole blood was studied 4, 24, 48, 72 and 96 hours after administration of the maximal injected dose, 1.0 mg/kg PrDChVP. NTE inhibition was measured in brain by the colorimetric assay and in whole blood using the amperometric biosensor assay (Figure 5). Figure 5 shows that NTE activity in brain and blood of hens killed 4, 24, 48, 72 and 96 hours after dosing with 1 mg/kg PrDChVP is reduced significantly in comparison to respective control values.

Discussion

This paper presents the overview of our previous and current work on development of amperometric sensors for NTE. We pioneered the application of biosensors for NTE assay using a Clark-type oxygen electrode covered with tyrosinase immobilized in polyvinyl alcohol (Figure 1, I).[38] A hen brain lyophilized NTE preparation[41,42] was used as a source of enzyme. The sensitivity of this biosensor did not exceed the sensitivity of the traditional colorimetric method. However, the tyrosinase carbon-paste electrode (Figure 1, II) improved the sensitivity of the NTE assay compared to the colorimetric method or an earlier amperometric technique based on oxygen detection.[29]

Due to the high sensitivity of the tyrosinase carbon-paste electrode, the influence of interfering blood components (ascorbic acid, tyrosine and others) was diminished by the extensive sample dilution (from 1:275 to 1:2200), thus allowing NTE to be detected selectively and with high sensitivity in whole blood, where the usual colorimetric assay is impossible.[29,52] Using phenol calibration of the MPMS-modified tyrosinase carbon paste electrode, blood NTE specific activity was calculated to be 0.25 nmol phenol produced/(min $\times$ mg protein) in agreement with the earlier data.[29] The minimum statistically significant decrease in NTE catalytic activity that can be detected with this method is 0.016 nmol phenol/(min $\times$ mg protein), when the volume of blood sample taken for analysis is 20 µL.

A linear dependence of the apparent NTE activity on blood concentration in analysed samples (the extent of blood dilution) (Figure 2) demonstrates that the influence of the interfering blood components on the phenol analysis is negligible. Furthermore, the influence of interfering electrochemical reactions on selectivity and sensitivity of the NTE assay with tyrosinase carbon paste electrodes is decreased by the relatively low operating potential (Figure 1, II, III). It should be noted that the MPMS-modified tyrosinase carbon paste electrode, in addition to having a high sensitivity, has high operational and functional stabilities[39] that enable improved reproducibility of measurements and prolonged ‘working life’ of the electrode.

The data presented in Table 1 show that consistent agreement was obtained between the colori-

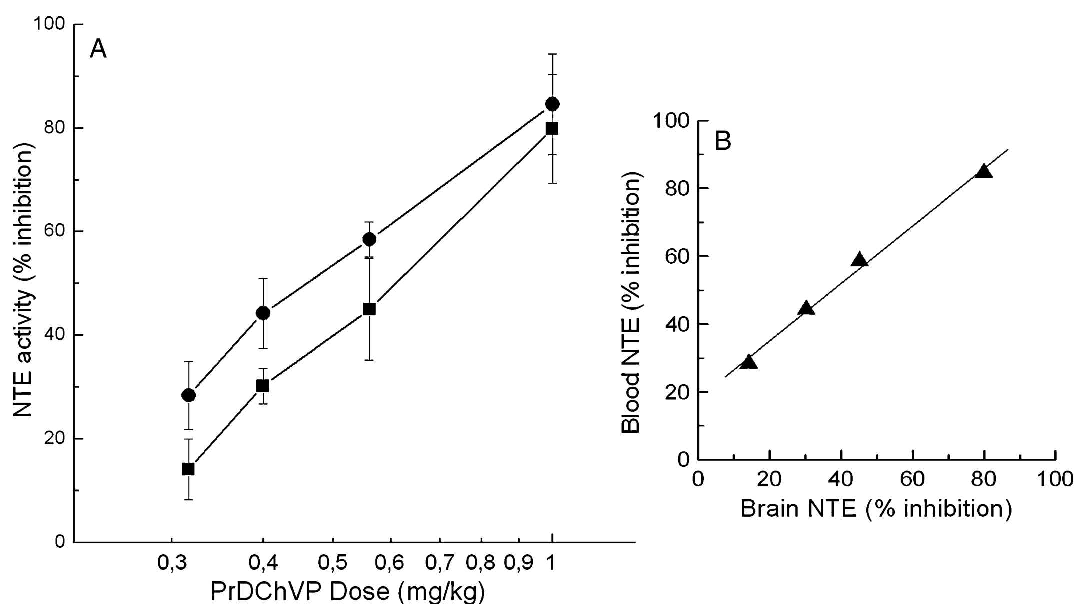


Figure 4 Dose-related NTE inhibition in brain and whole blood of hens 4 hours after injection of the neuropathic OP, PrDChVP (A) and correlation between blood and brain NTE inhibition (B). NTE assayed colorimetrically in brain and amperometrically (MPMS-modified tyrosinase carbon biosensor) in blood; both tissues assayed after freezing and thawing. Closed square (—■—), brain NTE; closed circle (—●—), blood NTE. Results are % control values for each tissue expressed as mean $\pm$ SEM, $n = 3$. Control NTE activities, nmol/(min $\times$ mg protein), mean $\pm$ SEM, $n = 3$: brain $= 21 \pm 2$, whole blood $= 0.23 \pm 0.02$.

Downloaded from http://het.sagepub.com at UNIV OF MICHIGAN on July 5, 2007
© 2007 SAGE Publications. All rights reserved. Not for commercial use or unauthorized distribution.

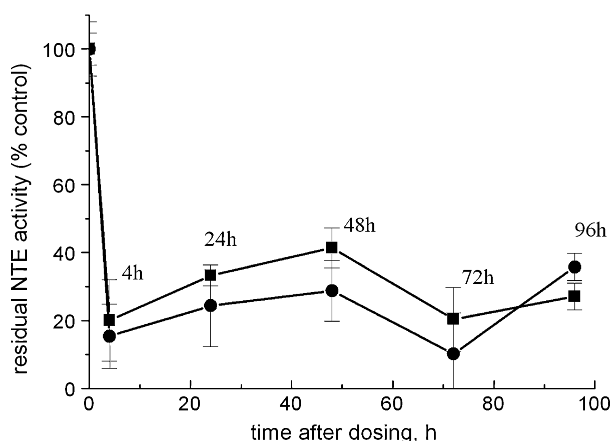


Figure 5 Time-dependence of NTE activity in brain and whole blood of hens injected with 1 mg/kg of the neuropathic OP, PrDChVP. NTE assayed colorimetrically in brain and amperometrically (MPMS-modified tyrosinase carbon biosensor) in blood; both tissues assayed after freezing and thawing. Closed square (—■—), brain NTE; closed circle (—●—), blood NTE. Results are % control values for each tissue expressed as mean ± SEM, $n = 3$. Control NTE activities, nmol/(min × mg protein), mean ± SEM, $n = 3$: brain = 21 ± 2, whole blood = 0.23 ± 0.02.

metric and amperometric methods for I_{50} determinations of mipafox against hen brain NTE, hen and human lymphocyte NTE, hen and human whole blood NTE, as well as PrDChVP against hen brain, hen blood and human blood NTE. In addition, the mipafox I_{50} values determined by the two methods for hen brain, hen and human lymphocyte NTE and PrDChVP I_{50} for hen brain overlap with those reported earlier in the literature. From these comparisons, it is apparent that PrDChVP is about 100-fold more potent than mipafox against lymphocyte or blood NTE, in agreement with data reported for brain NTE.[48] Table 1 also shows that a good agreement was obtained between the two amperometric methods (MPMS-modified and unmodified electrodes) for I_{50} determinations of mipafox and PrDChVP against blood NTE.

In order to use the measurement of blood NTE activity as a surrogate of brain NTE, the correlation between the inhibition of the enzyme in brain and blood should be known. Previous work has shown that assay of lymphocyte NTE provides a reliable monitor of exposure to neuropathic OPs within 24 hours between exposure and measurement.[25] To investigate the possibility of using blood NTE inhibition as a biochemical marker of neuropathic OP exposure, NTE inhibition in brain and lymphocytes (Experiment I) as well as in brain and blood (Experiment II) was studied 24 hours after dosing hens with the neuropathic OP, PrDChVP.[44] NTE activity in brain and lymphocytes was determined colorimetrically and in whole blood amperometrically using the tyrosinase carbon paste biosensor.

Data from both experiments are presented in Figure 3. Brain, lymphocyte and blood NTE were inhibited in a dose-responsive manner (linear trend, $P < 0.0001$) and showed a similar pattern and degree of inhibition. There were strong correlations of NTE inhibition between brain and lymphocytes ($r = 0.994$, $P < 0.0001$, $n = 6$), brain and blood ($r = 0.997$, $P = 0.003$, $n = 4$) and lymphocytes (I) and blood (II) ($r = 0.946$, $P = 0.003$, $n = 4$).[44] This analysis supports the use of whole blood NTE inhibition measured by the new biosensor method as a biochemical marker for exposure to neuropathic OPs. Furthermore, these results are in agreement with previous work using lymphocyte NTE as a biomarker within 24 hours of exposure.[25] In addition, the results indicate that whole blood NTE inhibition reflects NTE inhibition in brain within 24 hours of exposure.[44]

To evaluate further the dose and time dependence of the blood NTE assay, we studied NTE inhibition in brain and blood at a short time (4 hours) after dosing hens with PrDChVP (0.32–1.0 mg/kg, im), as well as 4, 24, 48, 72 and 96 hours after the maximal injected dose, 1.0 mg/kg. In both experiments, NTE inhibition was measured in brain by the colorimetric assay and in whole blood using the amperometric assay with the MPMS-modified tyrosinase carbon paste electrode.

Data from the first experiment are presented in Figure 4. Brain and blood NTE were inhibited in a dose-responsive manner 4 hours after dosing (linear trend, $P < 0.0001$; Figure 4A), and NTE inhibition was highly correlated between brain and blood ($r = 0.997$, $n = 4$; Figure 4B).

Data on time dependence of blood and brain NTE inhibition are presented in Figure 5. NTE activity in brain and blood of hens killed 4, 24, 48, 72 and 96 hours after dosing with 1 mg/kg PrDChVP differed significantly from respective control values ($P < 0.0001$, ANOVA, Dunnett's post-test). During all measured times, brain NTE was inhibited (mean ± SE, $n = 5$) 72 ± 4% and blood NTE 75 ± 3% relative to controls. The results demonstrate that whole blood NTE is a reliable biomarker of exposure to neuropathic OPs during 96 hours between exposure and measurement.

As noted above, previous work has shown that inhibition of lymphocyte NTE is a reliable monitor of exposure to neuropathic OPs when measured within 24 hours of exposure.[25] However, the same work showed that correlation of inhibition between lymphocyte and brain NTE 48 hours after exposure was poor, with consistently less inhibition of lymphocyte NTE relative to brain NTE. The reason for this difference in correlation between 24 and 48

Downloaded from http://het.sagepub.com at UNIV OF MICHIGAN on July 5, 2007
© 2007 SAGE Publications. All rights reserved. Not for commercial use or unauthorized distribution.

hours is unclear. The authors speculated that the divergence of brain and lymphocyte NTE inhibition at 48 hours was due to dissimilar kinetics of reappearance of enzyme activity in the two tissues.[25] In the brain, reappearance of NTE activity could occur only by resynthesis, whereas in lymphocytes, NTE activity could return via resynthesis and turnover of cells.

In the present work, good correlation between whole blood NTE and brain NTE was maintained for 96 hours. It should be noted that whole blood contains not only lymphocyte NTE, but also platelet NTE.[20,21] Moreover, the present work used a different compound (PrDChVP) and route of administration from those used in previously reported results.[25] The relationships between blood and brain NTE inhibition are likely to vary between different OPs with different toxicokinetic properties and between different routes of exposure. An extended examination is necessary in order to clarify these issues more fully.

In conclusion, the results support the validity of measurements carried out with the new biosensor method. The tyrosinase carbon-paste biosensors are suitable for assaying NTE in whole human and hen blood, which cannot be done using the classical colorimetric technique. Other advantages include the small sample volume required (100 μL), simplicity of sample preparation and rapid analysis time. A strong correlation between NTE inhibition in whole blood with that in brain suggests that the biosensor NTE assay for whole blood appears to be promising, not only as a biomarker of human exposure to neuropathic OP compounds, but also as a predictor of OPIDN and as an adjunct to its early diagnosis.

Application of the developed biosensors will enable rapid assessment of human exposure to neuropathic OPs. This is important in biomonitoring and epidemiological studies as well as in supporting consequence management and risk minimization of a chemical attack or chemical accident. Finally, because OPIDN depends not only upon inhibition of NTE, but also ageing of the NTE-OP conjugate, future work will investigate the use of the biosensors for measuring NTE ageing as well as inhibition.

Acknowledgements

This material is based upon work supported in part by the International Science and Technology Center (Projects #1055.2 and #3130), the US Civilian Research and Development Foundation for the Independent States of the Former Soviet Union (CRDF) (award number RB2-2035 and RB2-2488), and the US Army Research Laboratory and the US Army Research Office under grant number DAAD19-02-1-0388.

References

1 Davis CS, Richardson RJ. Organophosphorus compounds. In Spencer PS, Schaumburg HH eds. *Experimental and clinical neurotoxicology*. Williams & Wilkins, 1980: 527–544.
2 Richardson RJ. Organophosphate poisoning, delayed neurotoxicity. In Wexler P ed. *Encyclopedia of toxicology*, second edition, Volume 3 (4 vols). Elsevier, Ltd., 2005: 302–306.
3 Johnson MK. The target for initiation of delayed neurotoxicity by organophosphorus esters: biochemical studies and toxicological applications. In Hodgson E, Bend JR, Philpot RM eds. *Reviews in biochemical toxicology*, Volume 4. Elsevier, 1982: 141–212.
4 Makhaeva GF, Malygin VV, Martynov IV. Organophosphorus pesticide induced delayed neurotoxicity (Review). *Agrochemistry (Russian)* 1987; 12: 103–124.
5 Abou-Donia MB. Organophosphorus ester-induced delayed neurotoxicity. *Annu Rev Pharmacol Toxicol* 1981; **21**: 511–48.
6 Cole DC, Carpio F, Julian J, Léon N. Assessment of peripheral nerve function in an Ecuadorian rural population exposed to pesticides. *J Toxicol Environ Health A* 1998; **55**: 77–91.
7 Lotti M. Organophosphate-induced delayed polyneuropathy in humans: perspectives for biomonitoring. *Trends Pharmacol Sci* 1987; **81**: 176–77.
8 Glynn P. Neuropathy target esterase. *Biochem J* 1999; **344**: 625–31.
9 Huggins DJ, Richardson RJ. Brainstem axolemmal protein phosphorylation in vitro in hens dosed with di-1-butyl-2,2-dichlorovinyl phosphate. *J Toxicol Environ Health A* 1999; **56**: 263–82.
10 Johnson MK. Organophosphates and delayed neuropathy – is NTE alive and well? *Toxicol Appl Pharmacol* 1990; **102**: 385–99.
11 Randall JC, Yano BL, Richardson RJ. Potentiation of organophosphorus compound-induced delayed neurotoxicity (OPIDN) in the central and peripheral nervous system of the adult hen: distribution of axonal lesions. *J Toxicol Environ Health* 1997; **51**: 571–90.
12 Richardson RJ. Interactions of organophosphorus compounds with neurotoxic esterase. In Chambers JE, Levi PE eds. *Organophosphates: chemistry, fate, and effects.* Academic Press, 1992: 299–323.
13 Richardson RJ. Assessment of the neurotoxic potential of chlorpyrifos relative to other organophosphorus

Downloaded from http://het.sagepub.com at UNIV OF MICHIGAN on July 5, 2007
© 2007 SAGE Publications. All rights reserved. Not for commercial use or unauthorized distribution.

compounds: a critical review of the literature. *J Toxicol Environ Health* 1995; **44**: 135–65.
14 Barber D, Correll L, Ehrich M. Comparative effectiveness of organophosphorus protoxicant activating systems in neuroblastoma cells and brain homogenates. *J Toxicol Environ Health A* 1999; **57**: 63–74.
15 Weiner ML, Jortner BS. Organophosphate-induced delayed neurotoxicity of triarylphosphates. *Neurotoxicology* 1999; **20**: 653–73.
16 Moretto A. Experimental and clinical toxicology of anticholinesterase agents. *Toxicol Lett* 1998; **102/103**: 509–13.
17 Costa LG. Biomarker research in neurotoxicology: the role of mechanistic studies to bridge the gap between laboratory and epidemiological investigations. *Environ Health Perspect* 1996; **104** Suppl. 1: 55–67.
18 Bertoncin D, Russolo A, Caroldi S, Lotti M. Neuropathy target esterase in human lymphocytes. *Arch Environ Health* 1985; **40**: 221–30.
19 Dudek BR, Richardson RJ. Evidence for the existence of neurotoxic esterase in neuronal and lymphatic tissue of the adult hen. *Biochem Pharmacol* 1982; **31**: 1117–21.
20 Maroni M, Bleecker ML. Neuropathy target esterase in human lymphocytes and platelets. *J Appl Toxicol* 1986; **6**: 1–7.
21 Richardson RJ, Dudek BR. Neurotoxic esterase: characterization and potential for a predictive screen for exposure to neuropathic organophosphates. In Miyamoto J, Kearney PC eds. *Pesticide chemistry: human welfare and the environment*, Volume 3. Pergamon, 1983: 491–95.
22 Lotti M. Biological monitoring for organophosphate-induced delayed polyneuropathy. *Toxicol Lett* 1986; **33**: 167–72.
23 Lotti M, Becker CE, Aminoff MJ, Woodrow JE, Seiber JN, Talcott RE, Richardson RJ. Occupational exposure to the cotton defoliants DEF and merphos. A rational approach to monitoring organophosphorus-induced neurotoxicity. *J Occup Med* 1983; **25**: 517–22.
24 Lotti M, Moretto A, Zoppellari R, Dainese R, Rizzuto N, Barusco G. Inhibition of lymphocytic neuropathy target esterase predicts the development of organophosphate-induced delayed polyneuropathy. *Arch Toxicol* 1986; **59**: 176–79.
25 Schwab BW, Richardson RJ. Lymphocyte and brain neurotoxic esterase: dose and time dependence of inhibition in the hen examined with three organophosphorus esters. *Toxicol Appl Pharmacol* 1986; **83**: 1–9.
26 Ehrich M. Neurotoxic esterase inhibition: predictor of potential for organophosphate-induced delayed neuropathy. In Blancato JN, Brown RN, Dary CC, Saleh MA eds. *Biomarkers for agrochemicals and toxic substances. ACS Symposium Series 643.* American Chemical Society, 1996: 79–93.
27 Mutch E, Blain PG, Williams FM. Interindividual variations in enzymes controlling organophosphate toxicity in man. *Hum Exp Toxicol* 1992; **11**: 109–16.
28 Wilson BW, Henderson JD. Blood esterase determinations as markers of exposure. *Rev Environ Contam Toxicol* 1992; **128**: 55–69.
29 Sigolaeva LV, Makower A, Eremenko AV, Makhaeva GF, Malygin VV, Kurochkin IN, Scheller F. Bioelectrochemical analysis of neuropathy target esterase activity in blood. *Anal Biochem* 2001; **290**: 1–9.
30 Johnson MK. Improved assay of neurotoxic esterase for screening organophosphates for delayed neurotoxicity potential. *Arch Toxicol* 1977; **67**: 113–15.
31 Kayyali US, Moore TB, Randall JC, Richardson RJ. Neurotoxic esterase (NTE) assay: optimized conditions based on detergent-induced shifts in the phenol/4-aminoantipyrine chromophore spectrum. *J Anal Toxicol* 1991; **15**: 86–89.
32 Burton SG. Biocatalysis with polyphenol oxidase: a review. *Catal Today* 1994; **22**: 459–87.
33 Uchiyama S, Hasebe Y, Shimizu H, Ishihara H. Enzyme-based catechol sensor based on the cyclic reaction between catechol and 1,2-benzoquinone, using L-ascorbate and tyrosinase. *Anal Chim Acta* 1993; **276**: 341–45.
34 Makower A, Eremenko AV, Streffer K, Wollenberger U, Scheller FW. Tyrosinase-glucose dehydrogenase substrate recycling biosensor. Highly sensitive measurement of phenolic compounds. *J Chem Technol Biotechnol* 1996; **65**: 39–44.
35 Skladal P. Mushroom tyrosinase-modified carbon paste electrode as an amperometric biosensor for phenols. *Coll Czech Chem Commun* 1991; **56**: 1427–33.
36 Wang J, Lopez D. Amperometric biosensor for phenols based on a tyrosinase-graphite-epoxy biocomposite. *Analyst* 1994; **119**: 455–58.
37 Marko-Varga G, Emneus J, Gorton L, Ruzgas T. Development of enzyme-based amperometric sensors for the determination of phenolic compounds. *Trends Anal Biochem* 1995; **14**: 319–20.
38 Sigolaeva LV, Eremenko AV, Makower A, Makhaeva GF, Malygin VV, Kurochkin IN. A new approach for determination of neuropathy target esterase activity. *Chem Biol Interact* 1999; **119/120**: 559–65.
39 Sokolovskaya LG, Sigolaeva LV, Eremenko AV, Gachok IV, Makhaeva GF, Strakhova NN, Malygin VV, Richardson RJ, Kurochkin IN. Improved electrochemical analysis of neuropathy target esterase activity by a tyrosinase carbon paste electrode modified by 1-methoxyphenazine methosulfate. *Biotechnol Lett* 2005; **27**: 1211–18.
40 Richardson RJ, Davis CS, Johnson MK. Subcellular distribution of marker enzymes and of neurotoxic esterase in adult hen brain. *J Neurochem* 1979; **32**: 607–15.
41 Makhaeva GF, Malygin VV. A stable preparation of hen brain neuropathy target esterase for rapid biochemical assessment of neurotoxic potential of organophosphates. *Chem Biol Interact* 1999; **119/120**: 551–57.
42 Makhaeva GF, Filonenko IV, Yankovskaya VL, Fomicheva SB, Malygin VV. Comparative studies of *O*,*O*-dialkyl-*O*-chloromethylchloroformimino phosphates interaction with neuropathy target esterase and acetylcholinesterase. *Neurotoxicology* 1998; **19**: 623–28.
43 Nielsen H. Quantitative micro determination of proteins and peptides. *Acta Chem Scand* 1958; **12**: 38–43.
44 Makhaeva GF, Sigolaeva LV, Zhuravleva LV, Eremenko AV, Kurochkin IN, Malygin VV, Richardson RJ. Biosensor detection of neuropathy target esterase in whole blood as a biomarker of exposure to neuropathic organophosphorus compounds. *J Toxicol Environ Health A* 2003; **66**: 599–610.

Downloaded from http://het.sagepub.com at UNIV OF MICHIGAN on July 5, 2007
© 2007 SAGE Publications. All rights reserved. Not for commercial use or unauthorized distribution.

45 International recommendations (Ethical codex) for carrying out the experiments on animals and corresponding guidelines approved by the USSR and Rusian Federation authorities (Ministry of Education order #742) 2001. Retrieved from http://www.bio.msu.ru/etica.html.
46 Padilla S, Veronesi B. The relationships between neurological damage and neurotoxic esterase inhibition in rats acutely exposed to tri-*ortho*-cresyl phosphate. *Toxicol Appl Pharmacol* 1985; **78**: 78–87.
47 Makhaeva GF, Sigolaeva LV, Zhuravleva LV, Eremenko AV, Kurochkin IN, Richardson RJ, Malygin VV. Neuropathy target esterase in whole blood: biomarker for exposure to neuropathic organophosphorus compounds. *ASA Newsl* 2002; **02/05**: 16–21.
48 Lotti M, Johnson MK. Neurotoxicity of organophosphorus pesticides: Predictions can be based on *in vitro* studies with hen and human enzymes. *Arch Toxicol* 1978; **41**: 215–21.
49 Davis CS, Richardson RJ. Neurotoxic esterase: characterization of the solubilized enzyme and the conditions for its solubilization from chicken brain microsomal membranes with ionic, zwitterionic, or nonionic detergents. *Biochem Pharmacol* 1987; **36**: 1393–99.
50 Novak R, Padilla S. An in vitro comparison of rat and chicken brain neurotoxic esterase. *Fundam Appl Toxicol* 1986; **6**: 464–71.
51 Yoshida M, Wu SY, Casida JE. Reactivity and stereospecificity of neuropathy target esterase and alpha-chymotrypsin with 2-substituted-4H-1,3,2-benzodioxaphosphorin 2-oxides. *Toxicol Lett* 1994; **74**: 167–76.
52 Sigolaeva LV, Makower A, Eremenko AV, Makhaeva GF, Malygin VV, Kurochkin IV, Scheller F. Neuropathy target esterase activity in blood: biosensor analysis. *Neurotoxicology* 2000; **21**: 637–38.

Downloaded from http://het.sagepub.com at UNIV OF MICHIGAN on July 5, 2007
© 2007 SAGE Publications. All rights reserved. Not for commercial use or unauthorized distribution.