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Enclosure 1

BIOSENSOR DETECTION OF NEUROPATHY TARGET ESTERASE IN WHOLE BLOOD AS A BIOMARKER OF EXPOSURE TO NEUROPATHIC ORGANOPHOSPHORUS COMPOUNDS

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Neuropathy target esterase (NTE) is the target protein for neuropathic organophosphorus (OP) compounds that produce OP compound-induced delayed neurotoxicity (OPIDN). Inhibition/aging of brain NTE within hours of exposure predicts the potential for development of OPIDN in susceptible animal models. Lymphocyte NTE has also found limited use as a biomarker of human exposure to neuropathic OP compounds. Recently, a highly sensitive biosensor was developed for NTE activity using a tyrosinase carbon-paste electrode for amperometric detection of phenol produced by hydrolysis of the substrate, phenyl valerate. The I_{50} (20 min at 37 °C) for N,N'-di-2-propylphosphorodiamidofluoridate (mipafox) against hen lymphocyte NTE was $6.94 \pm 0.28 \mu\text{M}$ amperometrically and $6.02 \pm 0.71 \mu\text{M}$ colorimetrically. For O,O-di-1-propyl O-2,2-dichlorovinyl phosphate (PrDChVP), the I_{50} against hen brain NTE was $39 \pm 8 \text{ nM}$ amperometrically and $42 \pm 2 \text{ nM}$ colorimetrically. The biosensor enables NTE to be assayed in whole blood, whereas this cannot be done with the usual colorimetric method. Amperometrically, I_{50} values for PrDChVP against hen and human blood NTE were 66 ± 3 and $70 \pm 14 \text{ nM}$, respectively. To study the possibility of using blood NTE inhibition as a biochemical marker of neuropathic OP compound exposure, NTE activities in brain and lymphocytes as well in brain and blood were measured 24 h after dosing hens with PrDChVP. Brain, lymphocyte, and blood NTE were inhibited in a dose-responsive manner, and NTE inhibition was highly correlated between brain and lymphocyte ($r = .994$) and between brain and blood ($r = .997$). The results suggest that the biosensor NTE assay for whole blood could serve as a biomarker of exposure to neuropathic OP compounds as well as a predictor of OPIDN and an adjunct to its early diagnosis.

Organophosphorus compound-induced delayed neurotoxicity (OPIDN) is a distal degeneration of motor and sensory axons in spinal cord and peripheral nerves that occurs 1 to 3 wk after acute exposure to certain organophosphorus (OP) compounds. This disease presents as flaccid paralysis and sensory loss of the lower limbs with upper limb involvement in severe cases (Davis & Richardson, 1980; Richardson, 1998). Pathogenesis is independent of inhibition of acetylcholinesterase (AChE) and can be induced by OP compounds with low acute toxicity (Johnson, 1982; Makhaeva et al., 1987). Although OPIDN has occurred in epidemic proportions throughout the world, accidental episodes are currently rare (Abou-Donia, 1981; Richardson, 1998). Nevertheless, the high human susceptibility to OPIDN (Cole et al., 1998; Lotti, 1987), its insidious onset, and the usually permanent debilitating effects suggest the possibility of neuropathic OP compounds being used as agents for chemical terrorism. Defense against such agents requires sensitive and specific detection of them and their biological effects. Thus, the development of biomarkers of human exposures to neuropathic OP compounds is a vital component of a system of prediction and early diagnosis of OPIDN.

Considerable evidence points to a neuronal serine hydrolase, neuropathy target esterase (neurotoxic esterase, NTE), as the primary target molecule in OPIDN (Glynn, 1999; Huggins & Richardson, 1999). The disease is thought to be initiated by organophosphorylation of NTE with subsequent specific and rapid modification (aging) of the inhibited enzyme (Johnson, 1982, 1990; Randall et al., 1997; Richardson, 1992). There are species differences in sensitivity to OPIDN, for example, chickens > rats, and within a given species, adults are more sensitive than young animals (Richardson, 1995). Accordingly, the experimental animal model of choice for studies of OPIDN is the adult hen

(Barber et al., 1999; Weiner & Jortner, 1999). OPIDN in hens is associated with a threshold of >70% inhibition (and presumed attendant aging) of brain NTE after single exposures. The threshold in humans is not known, although available data suggest that it is comparable to that observed in hens (Moretto, 1998).

The relationship between NTE inhibition/aging and the development of OPIDN has the potential to be further exploited as a biomarker (Costa, 1996; Johnson, 1982; Richardson, 1992). Inhibition of brain NTE within hours of exposure to a neuropathic OP compound predicts the potential for OPIDN to appear in susceptible animal models (e.g., adult hens) after a delay of 1 to 3 wk. NTE has also been found in circulating lymphocytes and platelets (Bertoncin et al., 1985; Dudek & Richardson, 1982; Maroni & Bleecker, 1986; Richardson & Dudek, 1983), and lymphocyte NTE has been used or proposed for use as an accessible biomarker of animal and human exposure to neuropathic OP compounds (Johnson, 1990; Lotti, 1986; Lotti et al., 1983, 1986; Richardson & Dudek, 1983; Schwab & Richardson, 1986). Furthermore, lymphocyte NTE inhibition has been suggested as a predictor of OPIDN or an adjunct for its early diagnosis (Ehrich, 1996; Lotti, 1987; Mutch et al., 1992; Richardson & Dudek, 1983; Wilson & Henderson, 1992).

Despite the potential utility of lymphocyte NTE as a biomarker, the time, resources, and relatively high sample volumes required to separate blood components mitigate against using isolated lymphocytes routinely to monitor NTE activity. Thus, if the need were to arise to assess exposures of individuals to neuropathic OP compounds, it would be advantageous to be able to assay NTE in small volumes of whole blood (Sigolaeva et al., 2001).

Measurement of NTE activity has classically been done by colorimetric determination of phenol released by hydrolysis of the substrate phenyl valerate (Johnson, 1977; Kayyali et al., 1991). The absorbance maximum of the red phenol chromophore overlaps substantially with that of whole blood homogenates, and dilution of the blood to remove the interfering absorbance decreases NTE activity below the detection limit of the colorimetric assay (Sigolaeva et al., 2001). Thus, the colorimetric assay cannot be used to assay NTE in whole blood.

The problems inherent in a colorimetric NTE assay can be eliminated by using an amperometric technique to detect phenol. Recently, a new biosensor for NTE assay was introduced using a tyrosinase carbon-paste electrode to detect phenol produced by the hydrolysis of phenyl valerate. Data showed that the tyrosinase carbon-paste electrode improved the sensitivity of the NTE assay 10-fold compared to the colorimetric method or an earlier amperometric technique. Moreover, the new electrode operates in a flow-injection mode that requires only 2 to 3 min per analysis (Sigolaeva et al., 1999, 2000, 2001).

The present research was carried out to demonstrate further the utility of the new biosensor method for assaying NTE activity in whole blood and using these measurements as a biomarker of neuropathic OP compound exposure. The biosensor assay was used to obtain I_{50} values for inhibition of apparent NTE activity in vitro with the neuropathic OP compounds,

N,N'-di-2-propylphosphorodiamidofluoridate (mipafox) in hen lymphocytes, and *O,O*-di-1-propyl *O*-2,2-dichlorovinyl phosphate (PrDChVP) in hen brain, hen whole blood, and human whole blood. These values were compared with those obtained for hen brain and hen lymphocytes by colorimetry. In addition, experiments were conducted *in vivo* to investigate the dose-related inhibition of NTE 24 h after administration of PrDChVP to adult hens. NTE activity in brain and lymphocytes was determined colorimetrically and in whole blood amperometrically using the new biosensor method. Correlations were then examined between NTE inhibition in brain and lymphocytes, and in brain and blood.

MATERIALS AND METHODS

Chemicals

Phenyl valerate (PV), *N,N'*-di-2-propylphosphorodiamidofluoridate (mipafox), and *O,O*-di-1-propyl *O*-2,2-dichlorovinyl phosphate (PrDChVP) were synthesized and characterized in the Institute of Physiologically Active Compounds, Russian Academy of Sciences (Russia), and in the Institute of Organic Chemistry, Ukrainian Academy of Sciences (Ukraine). The purity of all substances was >99% (by spectral, chromatographic, and elemental analysis data). Mushroom tyrosinase (monophenol monooxidase, EC 1.14.18.1, activity 3800 U/mg for L-tyrosine), graphite powder, *O,O*-diethyl *O*-4-nitrophenylphosphate (paraoxon), 4-aminoantipyrine, phenol, and potassium ferricyanide were from Sigma Chemical Co. (St. Louis, MO). The Coomassie protein kit was from Pierce Chemical Co. (Rockford, IL). All other chemicals were analytical grade and used without further purification. Aqueous solutions were prepared using deionized water.

Tyrosinase Carbon-Paste Electrode and Biosensor

The tyrosinase electrode was prepared as described previously (Sigolaeva et al., 2001). All measurements were performed with an applied potential of -150 mV versus Ag/AgCl. Current was measured by an IPC2000 potentiostat coupled to a computer (Institute of Physical Chemistry, Russian Academy of Sciences, Moscow, Russia). Operation of the device and processing of electrochemical measurements were done using software developed in the laboratory.

Studies In Vitro

Hen Brain NTE A lyophilized hen brain membrane fraction consisting of combined mitochondrial/synaptosomal and microsomal pellets ($P_2 + P_3$) (Richardson et al., 1979) preinhibited with paraoxon ($40 \mu\text{M}$ at 25°C for 45 min) was used as a source of NTE. It was prepared as described (Makhaeva & Malygin, 1999; Makhaeva et al., 1998) and stored in sealed ampoules. Before use, ampoule contents were suspended with a glass/glass Potter homogenizer in 2 ml of working buffer (50 mM Tris-HCl, 0.2 mM ethylenediamine tetraacetic

acid [EDTA], pH 8.0, at 25 °C). The preparations of neuronal NTE have a specific activity of about 40 nmol/(min×mg protein).

Isolation of Lymphocytes From Hen Blood Lymphocytes were isolated as described (Schwab & Richardson, 1986) by centrifuging in a Ficoll-Verografin density gradient. The lymphocyte fraction was resuspended in working buffer to make a concentration equivalent to 2×10^7 to 4×10^7 cells/ml and sonicated (10s, power output 50 W) before NTE assay.

Whole Blood Preparation Whole human or hen blood stabilized by citrate/EDTA was diluted 10-fold with working buffer and homogenized in a Potter glass/glass homogenizer before NTE assay.

Sample Preparation for Colorimetric or Electrochemical (Biosensor) NTE Assay NTE activity was determined according to the differential inhibition method of Johnson (1977) as a microassay version (Escudero et al., 1996) with slight modifications (Kayyali et al., 1991). Prepared as already described, samples of sonicated lymphocytes (40 µl) or whole blood diluted with working buffer (200 µl) were incubated at 37 °C with 50 µM paraoxon for 20 min (sample B) or with 50 µM paraoxon plus 250 µM mipafox for 20 min (sample C) in a final volume of 400 µl. PV was then added. After incubating for 30 min at 37 °C, the reaction was stopped by addition of aqueous sodium dodecyl sulfate (SDS). Enzymatically released phenol was assayed colorimetrically or amperometrically (biosensor). In the case of the paraoxon-preinhibited preparation of hen brain NTE, samples (20 µl) were incubated in working buffer (sample B) or in working buffer with 250 µM mipafox (sample C) in a final volume of 400 µl for 20 min at 37 °C, followed by incubation with PV and termination of the reaction by SDS as described earlier.

Colorimetric Phenol Assay Phenol released by the enzymatic hydrolysis of PV was assayed in 96-well microtiter plates at 492 nm using an Anthos HT1 microplate absorption photometer (Anthos Labtec Instruments GmbH, Salzburg, Austria) after incubation of 100 µl of final reaction mixture at room temperature for 10 to 15 min with 4-aminoantipyrine and potassium ferricyanide to develop the chromophore (Escudero et al., 1996; Johnson, 1977; Kayyali et al., 1991). Each measurement was made in triplicate. The absorbance difference between samples B and C was used for calculation of NTE activity using a phenol-standard calibration curve.

Amperometric (Biosensor) Phenol Assay Prior to measurements, samples prepared as already described were further diluted 10- to 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 duplicate. The concentration of phenol was determined according to a phenol calibration curve obtained under the same conditions. The difference in the analytical signals found between samples B and C was used for calculation of NTE activity.

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 to 12 different concentrations of either mipafox from 10^{-9} to 10^{-3} M or PrDChVP from 10^{-11} to 10^{-5} M for 20 min at 37°C, in working buffer. Residual NTE activity was then determined using either the colorimetric or the amperometric (biosensor) method (see earlier description). Each measurement was made in triplicate (colorimetry) or in duplicate (amperometry). I_{50} values were calculated as described by Sigolaeva et al. (2001). Every value represents the mean $\pm$ SEM from three independent experiments.

Protein Assay Protein was determined using a Coomassie protein assay kit from Pierce Chemical Co. (Rockford, IL) with bovine serum albumin as a reference standard.

Studies In Vivo

Animals Adult white Leghorn hens (18 mo of age, 1.5 to 2.0 kg) were from Noginsk poultry farm (Noginsk, Russia). Hens were kept three to a cage with food and tap water ad libitum. Hens were kept in a room with a 12-h light cycle and controlled temperature (20 to 23°C).

Dosing Experiment I 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, 1.0, 1.6, and 2.2 mg/kg to groups of 3 hens per dose to assess inhibition of NTE in brain and lymphocytes 24 h after administration. All hens were pretreated with atropine sulfate, 20 mg/kg in water, injected im (1.0 ml/kg) 20 min before PrDChVP injection. Control animals received atropine sulfate only. At 24 h after PrDChVP administration, hens were decapitated and blood was collected immediately in heparinized plastic vials. Heparin was added to a concentration of 20 units/ml and the blood was diluted 1.0/1.5 (v/v) with balanced salt solution (0.001% D-glucose, 5.0 mM CaCl_2 , 98 mM MgCl_2 , 14.5 mM Tris, 126 mM NaCl, pH 7.6).

Lymphocyte NTE Lymphocytes were isolated as described (Schwab & Richardson, 1986) by centrifuging in a Ficoll–Verografin density gradient. The lymphocyte fraction was resuspended in working buffer to make a concentration equivalent to 2×10^7 to 4×10^7 cells/ml and sonicated (10 s, power output 50 W) before NTE assay by the colorimetric method of Johnson (1977) using a 40-min incubation with PV.

Brain NTE Each brain was rapidly removed, homogenized at 4°C in 5 volumes of working buffer with a Potter homogenizer, and centrifuged for 15 min at $9000 \times g$ at 4°C. The brain 9S supernatant was used for NTE assay (Padilla & Veronesi, 1985) using the colorimetric method of Johnson (1977).

Protein Assay Protein concentrations were determined by the microbiuret method (Nielsen, 1958) using bovine serum albumin as a standard.

Dosing Experiment II PrDChVP in acetone/water, 1/1 (v/v), was injected im (1.0 ml/kg) into hens at 4 doses chosen from dosing experiment I—0.32, 0.40, 0.56, and 1.0 mg/kg—to groups of 3 hens per dose to assess

NTE inhibition in brain and whole blood 24 h after administration. All hens were pretreated with atropine sulfate in water, 20 mg/kg im (1.0 ml/kg), 20 min before PrDChVP injection. Control animals received atropine sulfate only. At 24 h after PrDChVP administration, hens were decapitated and 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/ml blood), frozen in liquid nitrogen, and stored at -20°C prior to NTE assay.

Whole Blood NTE Assay Frozen blood samples were allowed to thaw to room temperature and assayed for NTE activity using the tyrosinase carbon-paste electrode amperometric method (biosensor method) as already described for in vitro methods.

Brain NTE Brains were rapidly removed, frozen in liquid nitrogen, weighed, and stored at -20°C before NTE assay. After being allowed to thaw at room temperature, the 9S supernatants were prepared and used for colorimetric determination of NTE as described earlier.

Statistical Analysis

In Vitro Studies All values are expressed as the mean $\pm$ 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 $p < .05$. 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, lymphocytes, 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 percent inhibition relative to control. The correlation of NTE inhibition between brain and lymphocytes (dosing experiment I) and brain and blood (dosing experiment II) was examined by plotting correlation curves and calculating associated correlation coefficients (r) and p values, using Origin 5.0 software, OriginLab Corp. (Northampton, MA).

RESULTS

Mipaflox and PrDChVP I_{50} Values Against NTE From Different Sources by Colorimetry and Amperometry

Mipaflox I_{50} values for hen lymphocyte NTE were $6.94 \pm 0.28 \mu\text{M}$ amperometrically and $6.02 \pm 0.71 \mu\text{M}$ colorimetrically ($p > .05$). For PrDChVP, I_{50} values for hen brain NTE were $39 \pm 8 \text{ nM}$ amperometrically and $42 \pm 2 \text{ nM}$ colorimetrically ($p > .05$). Amperometrically, PrDChVP I_{50} values for hen and human blood NTE were $66 \pm 3 \text{ nM}$ and $70 \pm 14 \text{ nM}$, respectively ($p > .05$).

Studies In Vivo

Dosing Experiment I Inhibition of NTE in hen brain and lymphocytes was studied 24 h after injecting hens with increasing doses of the neuropathic OP compound, PrDChVP. NTE inhibition was measured in brain and lymphocytes using the colorimetric assay for both tissues (Figure 1).

Dosing Experiment II Inhibition of NTE in hen brain and whole blood was studied 24 h 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 1).

DISCUSSION AND CONCLUSION

Consistent agreement was obtained between the colorimetric and amperometric methods for I_{50} determinations of mipafox against hen lymphocyte NTE and PrDChVP against hen brain, hen blood, and human blood NTE. In

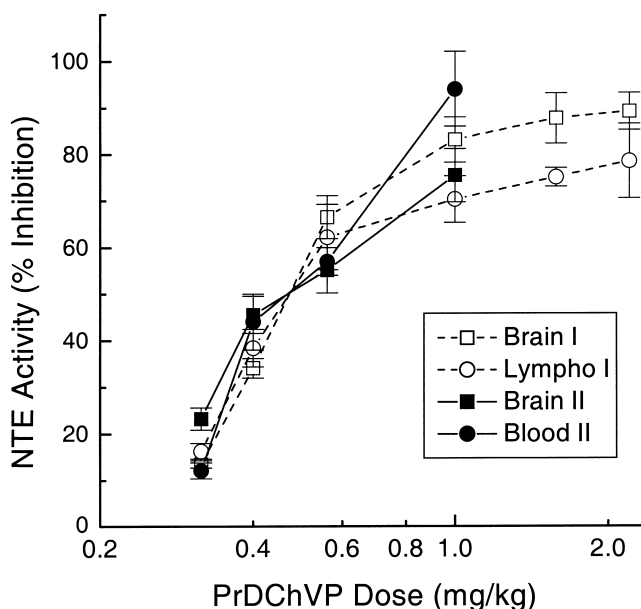


FIGURE 1. Dose-related NTE inhibition in brain, lymphocytes, and whole blood of hens 24 h after injection of the neuropathic OP compound, *O,O*-di-1-propyl *O*-2,2-dichlorovinyl phosphate (PrDChVP). Results are percent of control values for each tissue expressed as mean $\pm$ SEM, $n = 3$. Open square, dashed line = brain NTE, dosing experiment I (NTE assayed colorimetrically in brain and lymphocytes; both tissues assayed fresh). Open circle, dashed line = lymphocyte NTE, experiment I. Closed square, solid line = 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 NTE, dosing experiment II. Control NTE activities, nmol/(min $\times$ mg protein), mean $\pm$ 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 .

addition, the mipafox I_{50} values determined by the two methods for lymphocyte NTE overlap with those reported earlier for human lymphocytes, as well as with the value determined amperometrically for human whole blood (Sigolaeva et al., 2001). 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 (Lotti & Johnson, 1978).

During the development of the biosensor technique for NTE measurement in whole blood, the influence of potentially interfering biological materials on the phenol assay was studied (Sigolaeva et al., 2001). A number of phenolic compounds present in biological samples can be substrates for the immobilized tyrosinase, and the corresponding analytical responses cannot be ignored. It was found that contact of homogenized hen and human whole blood with the electrode surface does not lead to any notable contamination of the electrode, and only a high content of biological material in the analyte affected the phenol assay significantly. The sensitivity of the electrode was sufficient, however, to measure phenol in samples that had been diluted 100- to 200-fold in order to minimize the influence of interfering substances on the phenol signal (Sigolaeva et al., 2001).

In order to use the measurement of blood NTE activity as a mirror of brain NTE, the correlation between the inhibition of the enzyme in brain and blood should be known. Assay of lymphocyte NTE was shown to provide a reliable monitor of exposure to neuropathic OP compounds within 24 h between exposure and measurement (Schwab & Richardson, 1986). To study the possibility of using blood NTE inhibition as a biochemical marker of neuropathic OP exposure and its correlation with brain NTE inhibition, two dosing experiments were carried out. In both experiments, NTE inhibition was measured 24 h after injecting hens with the neuropathic OP compound, PrDChVP. In dosing experiment I, NTE inhibition was measured in both brain and lymphocytes by the colorimetric assay. In dosing experiment II, NTE inhibition was measured in brain by the colorimetric assay and in whole blood using the amperometric biosensor assay. Data from both experiments are presented in Figure 1, showing a similar pattern and degree of dose-responsive inhibition of brain, lymphocyte, and whole blood NTE.

Data replotted and analyzed from Figure 1 show strong correlations ($r > .99$) of NTE inhibition between brain and lymphocytes (Figure 2A) and brain and whole blood (Figure 2B). Moreover, a strong correlation ($r = .946$, $p = .00267$, $n = 4$) was found between inhibition of lymphocyte NTE (dosing experiment I) and blood NTE (dosing experiment II), as well as between brain NTE inhibitions in dosing experiments I and II ($r = .968$, $p = .0322$, $n = 4$). Thus, the results indicate a high degree of correspondence between the two experiments. This analysis further supports the use of whole blood NTE inhibition measured by the new biosensor method as a biochemical marker for exposure to neuropathic OP compounds. Furthermore, these results indicate that whole blood NTE inhibition reflects NTE inhibition in brain.

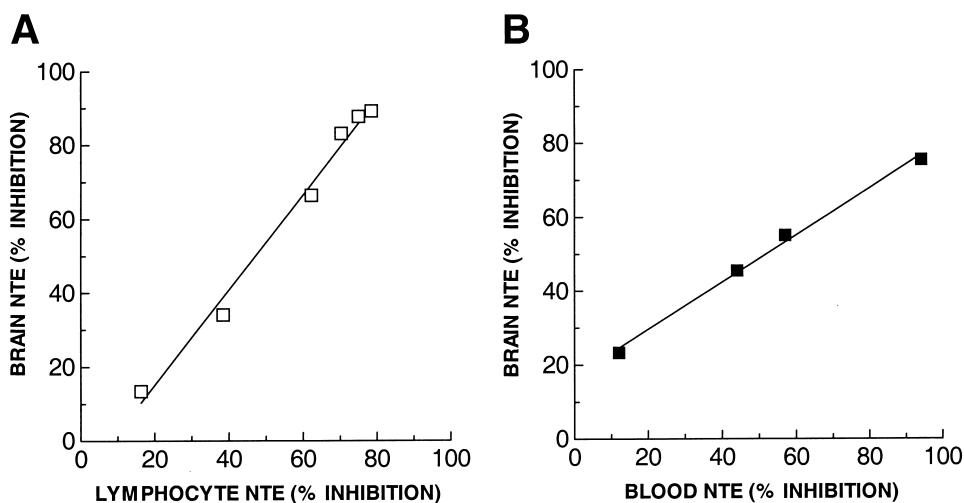


FIGURE 2. Correlations of NTE inhibition (data from Figure 1) between the following tissues in hens dosed with the neuropathic OP compound, *O,O*-di-1-propyl *O*-2,2-dichlorovinyl phosphate (PrDChVP): (A) brain and lymphocytes (dosing experiment I, NTE assayed colorimetrically in both tissues) ($r = .994$, $p < .0001$, $n = 6$); (B) brain and whole blood (dosing experiment II, NTE assayed colorimetrically in brain and amperometrically in blood) ($r = .997$, $p = .00267$, $n = 4$).

In conclusion, the data presented and discussed above support the validity of measurements carried out with the new biosensor method. The tyrosinase carbon-paste biosensor is 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, simplicity of sample preparation, rapid analysis time, and strong correlation between NTE inhibition in whole blood with that in brain. Thus, the biosensor NTE assay for whole blood appears to be promising, not only as a biomarker of human exposure to neuropathic OP compounds, but as a predictor of OPIDN and an adjunct to its early diagnosis.

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