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Project Summary

Development of an *In Vitro* Neurotoxicity Assay

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The aim of this project was to investigate the development of a totally *in vitro* neurotoxicity assay system using the enzyme neurotoxic esterase. Such a system was sought to allow rapid assessment of a large number of compounds for neurotoxicity.

In addition to a thorough biochemical characterization of several tissues containing neurotoxic esterase, a phylogenetic study of NTE occurrence was conducted. This search was made to identify sources of NTE for study.

Purified or partially purified neurotoxic esterase was to be immobilized on a solid matrix for eventual use in a continuous flow reactor.

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

One of the major concerns of the U.S. Environmental Protection Agency's Health Effects Research Laboratory is the testing and assessment of chemical pesticides for toxicity. Of particular concern are widely used pesticide compounds that may adversely affect human health.

Many organophosphorus (OP) compounds are well suited for use as pesticides because of their natural anticholinesterase activity. However, these compounds, as well as industrially used OP compounds without anticholinesterase activity, can produce a delayed neurotoxic effect resulting in the distal degeneration of nerve axons. This often irreversible effect, characterized by concomitant ataxia and paralysis, is called organophosphorus distal axonopathy (OPDA).

Neurotoxic esterase (NTE) has been identified as the enzymatic site of the primary biochemical lesion observed in OPDA. This membrane-bound phenyl valerate hydrolase is found in the neural tissue of the adult hen. The neurotoxic potential of organophosphorous compounds is thus often predicted by dosing adult hens *in vivo* and assaying brain NTE activity after a suitable interval (usually 24 hours).

This study was undertaken by the University of Michigan in support of the Health Effects Research Laboratory's research goal of developing reliable, yet rapid and inexpensive, *in vitro* methods for assaying chemicals. Specifically, the study was initiated to determine the feasibility of developing an *in vitro* assay system employing isolated NTE as an indicator of neurotoxicity. This system was based on the differential sensitivity of phenyl valerate hydrolyzing esterase to neurotoxic and non-neurotoxic inhibitors. Using the assay, a compound (i.e., inhibitor) was designated "neurotoxic" strictly on the basis of its ability to produce OPDA. That is, even if it produced acute neurotoxicity, it was called "non-neurotoxic" unless it produced the specific syndrome of OPDA.

The development of an effective *in vitro* system required that a thorough characterization of NTE be made and that potential sources of NTE be identified. Previously, the presence of NTE

had been established only for hen brain. To locate and characterize other NTE sources, several tissue types from hens, chicks, rats, and humans suspected of having NTE activity were assayed. In addition, a phylogenetic search for NTE occurrence was conducted. (This search was not conducted to find sources of NTE for this study, but more for general informational purposes.)

For the tissues chosen, biochemical characterization of NTE activity was achieved by generating and comparing inhibitor titration curves and by determining inhibitor I_{50} values, substrate hydrolysis ratios, thermal inactivation curves, and pH profiles. These parameters were used to assess the similarity of NTE activity from different sources or after different treatments, such as solubilization and immobilization. To assess the relative merit of different NTE preparations for eventual use as a component in the *in vitro* test system, additional measurements of stability were made under various conditions of medium composition and temperature.

Complete neurotoxic and non-neurotoxic inhibitor titration curves were done for the following tissues of the hen: brain, spinal cord, peripheral nerve, red muscle, white muscle, heart, liver, kidney, spleen, and lymphocytes isolated from spleen and blood. Titration curves were also generated for chick, rat, and human brain lymphocytes and platelets.

For hen and chick brain tissue characterizations, homogenates were prepared from the brain tissue of White Leghorn "spent" hens and White Leghorn chicks. Paired samples of tissue homogenate were preincubated in buffer with either a non-neurotoxic inhibitor (paraoxon) or paraoxon and a neurotoxic inhibitor (mipafox). After preincubation, a phenyl valerate dispersion was added to the solution and vortexed. Following an antipyrene dye reaction, the NTE activity was determined by measuring the difference in absorbance between the two conditions (i.e., paraoxon and paraoxon + mipafox). This assay was modified as required for individual experiments or for use of tissues other than brain (e.g., lymphocytes and platelets).

Paraoxon titration curves were generated by varying the paraoxon concentration in the preincubation mixture over a concentration range. For tissues that showed very high phenyl valerate esterase activity at low inhibitor concentrations, an aliquot of the preincubation mixture was used for incubation with phenyl valerate. For each tissue studied, inspection of the paraoxon titration curve, together with consideration of the absolute activity remaining at the higher paraoxon concentration, yielded an optimum paraoxon concentration for use in the mipafox titration.

Mipafox titration curves were generated by including paraoxon in the preincubation medium (at the selected optimum concentration) and varying mipafox over a concentration range.

When working with lymphocytes isolated from spleen and blood, the assay and reagent volumes were reduced by a factor of 20 to accommodate the small amounts of tissue available. For peripheral nerve and small quantities of brain tissue, assay and reagent volumes were reduced by a factor of 2.

Results of the phylogenetic search for NTE activity showed NTE to be present in the neural tissue of birds, fish, reptiles, amphibians, and mammals whose ancestors evolved in the last 400 million years. Sheep, steer, lizard, newt, skate, shark, and sea lamprey were examined. Apparent NTE activity was found in all of these species, with the possible exceptions of skate and shark. No evolutionary pattern was discernible from the species examined.

Paraoxon and mipafox preincubated in hen brain homogenate produced titration curves which confirmed the presence of NTE in hen brain. The presence of NTE in spinal cord and peripheral nerve tissue was also confirmed.

No NTE activity was found for red muscle, white muscle, liver, or kidney tissue in the hen. For heart muscle, NTE activity was tentatively identified at 14% that of brain on a per gram wet weight basis. Spleen and blood lymphocyte curves were very similar to brain curves, and I_{50} values were also similar to brain I_{50} values using paraoxon.

Differential titration curves for human lymphocytes and platelets were very similar to those obtained from hen brain and lymphocytes, and showed the same pI_{50} for mipafox. Rat brain tissue was shown to have only about half the NTE activity of hen brain. NTE was relatively plentiful in both the lymphocytes and platelets of the human.

In order to compare the properties of the NTE obtained from different sources, the following enzymological characteristics were used: heat denaturation, pH-activity relationship, substrate specificity, and inhibitor specificity. Lymphocyte, platelet, and brain phenyl valerate hydrolases from hens, rats, and humans were compared with respect to these characteristics.

Preliminary heat denaturation experiments showed 55°C to be optimal for studies using hen NTE. For rat enzyme, 50°C was used. Brain and lymphocyte homogenates were preincubated at these temperatures, then diluted with buffer. Aliquots of these dilutions were assayed for NTE activity; brain homogenate was also assayed for anticholinesterase.

Hen lymphocyte and brain NTE denatured at similar rates. However, acetylcholinesterase denatured at a different rate, indicating that NTE denaturation is not simply due to general denaturation of proteins at this temperature. Rat NTE denatured very rapidly at 55°C, differing significantly from the hen enzyme. A similar increased heat sensitivity was not observed in the chick. Thus, either this characteristic is not related to both species' resistance to OPDA, or the species and age resistance are due to distinct mechanisms.

Hydrolysis ratios were used to infer that the activity in two tissue homogenates was attributable to the same enzyme(s). The hydrolysis ratio, at a specific inhibitor concentration, was calculated by dividing the specific activity obtained for phenyl valerate by that obtained for phenyl phenylacetate. This ratio was plotted against mipafox or diisopropyl fluorophosphate (DFP) concentration. The ratios for each tissue at each inhibitor concentration were quite similar, indicating that the hydrolase activity in these tissues was attributable to the same enzyme. A striking feature of the data is that these enzymes behave similarly in the two tissues with respect to the two inhibitors, and moreover, the ratio of substrate hydrolysis changed in the same way for both tissues.

Since the tissue distribution portion of the study revealed the presence of NTE in peripheral lymphocytes, and this enzyme had biochemical characteristics very similar to NTE in hen brain, the possibility of using lymphocyte NTE activity as an indicator of central nervous system NTE activity was explored. Dose-response curves were generated from brain and lymphocyte tissues dosed orally with tri-*ortho*-cresyl phosphate (TOCP). The correlation coefficient for a plot of percent NTE inhibition in brain versus lymphocytes was 0.92. This suggested that lymphocyte NTE could

be used to monitor brain NTE activity and detect neurotoxic exposure in the hen.

This correlation was expanded to include a variety of other neurotoxic and non-neurotoxic compounds. After initial experiments, 4 h post-dose was adopted as the time before assay. The response of brain and lymphocyte NTE activity at 4 h post dose to the other neurotoxic compounds chosen to expand the correlation gave a correlation coefficient for a plot of percent inhibition of lymphocyte NTE versus percent inhibition of brain NTE at 0.85. This result showed that lymphocyte NTE was a good monitor of brain NTE and could be used as a biomonitor of delayed neurotoxicity exposure and potential in hens.

However, the data obtained did not demonstrate conclusively that hen brain NTE and the enzyme operationally defined as NTE in lymphocytes were identical. Dosing with non-neurotoxic compounds caused a stimulation of NTE activity, i.e., there was a dichotomy of response in lymphocyte NTE to neurotoxic and non-neurotoxic compounds. Proof that the enzymes are the same must await further work on isolation of the enzyme from both tissue sources. However, the similarities in response to heat, pH, substrates, and inhibitors *in vitro*, as well as a similar response to neurotoxic OP compounds 4 h after dosing, support the idea that NTE exists in lymphocytes as well as in neural tissue and can be used as an indicator of neurotoxicity.

In order to obtain NTE for solubilization, and ultimately for immobilization, subcellular fractionation of hen brain was carried out. Up to 70% of the NTE in brain was recovered in the microsomal fraction (P_3). The objective of the solubilization experiments was to maximize the amount of NTE solubilized, while minimizing NTE activity lost from detergent inactivation.

Once optimal solubilization conditions were determined through stability studies, microsomal NTE was obtained for use in solubilization experiments. All fractions—microsomes, microsomes plus detergent, a supernatant obtained by microsome-detergent centrifuging, and a microsomal pellet—were assayed for NTE activity. Inhibitor characteristics, pH activity, and pH denaturation were investigated. Studies were conducted in a systematic manner, maintaining strict attention to detergent/protein ratios.

Ovine and bovine brain were investigated as starting material for the preparation of large quantities of soluble NTE. Brain homogenates from these species had approximately one-half the specific activity of hen brain. Activity was solubilized from both sources with unbuffered Triton X-100 according to the method used for hen brain. The ovine preparation was found to be very unstable, with half-life of 4-8 days at 4°C. The solubilized bovine preparation had a half-life of about 12 days at 4°C. An attempt was made to expedite the isolation of bovine brain microsomes by isoelectric precipitation with citrate. Using this technique, 60-80% of the activity could be recovered, but the activity could no longer be solubilized with detergent.

Immobilization of NTE was attempted on a variety of supports using several different binding methods. The most extensive studies were carried out on ion-exchange resins, using ionic binding. Immobilization was also attempted by means of hydrophobic binding to a phenoxyacetyl cellulose support and by three different methods of covalent bonding.

NTE was successfully immobilized with retention of catalytic activity by both covalent and ionic bonding. However, neither of these preparations equaled the stability of the free enzyme stored under comparable conditions. Both forms of immobilized NTE were obtained with sufficient activity on the matrix to be useful as an enzyme preparation. The stability of the ionically bound form is adequate for storage over about one week. This form reacts normally in the NTE assay and retains the inhibitor characteristics of the native enzyme.

Conclusions

Brain tissue from a wide variety of primitive and advanced species was found to possess apparent NTE activity, with the possible exception of shark and skate, where mipafox I_{50} values were shifted to values approximately 10-fold higher than found in hen brain. Chick and hen brain were found to have comparable activity and were identical by all characterization parameters used.

A major new finding was the existence of NTE in hen, rat, and human lymphocytes and in rat and human platelets. By all criteria used to characterize enzymatic activity, hen lymphocyte and brain NTE were identical. Moreover, the activity responded similarly after dosing with neurotoxic OP compounds, if measurements were carried out at 4 h post dosing. At 24 h, some compounds did not produce similar responses on the brain and lymphocyte enzymes. Lack of correlation at longer times may be due to the dynamics of the lymphocyte pool compared with the relatively stable situation in brain.

The best solubilization preparations proved to be from hen or chick brain using Triton X-100 detergent. Incorporation of a lipid mixture, asolectin, into the solubilization step was found to prevent loss of activity, indicating that NTE is a lipid-dependent enzyme. Solubilized NTE retained all the characteristics of native enzyme. This is an encouraging result in terms of further work on the isolation and use of NTE in an *in vitro* test system. Brain from larger species (ovine; bovine) conferred no advantage over chicken brain as a starting material for solubilization. These species gave activity that was relatively less stable or that differed in inhibitor characteristics from native chicken brain NTE.

A considerable quantity of detailed information on stability of NTE under various conditions was compiled in this study. In terms of the most promising preparation for further development of purification and an *in vitro* test system, the best conditions for storage appeared to be Triton X-100 solubilized NTE from chicken brain microsomes, stored unbuffered at -18°C.

During immobilization studies, covalent attachment to matrices resulted in extensive inactivation of the enzyme. The optimum immobilization system to date is binding to Triton X-100 solubilized NTE from chicken brain to DEAE-Sephacel™. The bound enzyme retains the inhibitor characteristics of native enzyme. However, the immobilized enzyme was not as stable as the soluble form under identical storage conditions.

Recommendations

Since chick and hen brain were found to have comparable activity, and to be identical by all characterization parameters used, it is recommended that chick brain be used as a tissue source for future work on the isolation of pure NTE.

Further research is also needed to determine the reason why at 24 h some compounds do not produce similar responses on brain and lymphocyte NTE.

Overall, it is recommended that efforts be continued to develop an *in vitro* assay system based on NTE. Because of the lack of stability of immobilized forms of

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the enzyme, it may still be necessary to perform assays in the batch mode. Emphasis should be placed on obtaining pure NTE in order to obviate the differential character of the assay. Also, further attention should be directed toward convenient ways to assess the aging reaction, which is the second element, in addition to simple inhibition, required to have neurotoxicity.

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***Ronald L. Baron** is the EPA Project Officer (see below).*
The complete report, entitled "Development of an In Vitro *Neurotoxicity Assay," (Order No. PB 81-208 159; Cost: $11.00, subject to change) will be available only from:*
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