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# Organ distribution of neuropathy target esterase in man*

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Some organophosphorous esters (OP)† induce a delayed polyneuropathy (OPIDP) in man and other susceptible species [1, 2]. The molecular target for the initiation of OPIDP is a protein called Neuropathy Target Esterase (NTE) [3]. The progressive phosphorylation of NTE in the nervous system soon after dosing represents the initial reaction [4–6] which is followed by a molecular rearrangement of the phosphorylated protein called "aging". This non-enzymatic cleavage of an alkyl group, generates a charged monosubstituted phosphoric acid residue on the protein [7–9]. It has also been shown in the hen, the animal of choice for OPIDP studies, that the threshold for initiation of this toxic response is 70–75% inhibition of axonal NTE [10, 11] and that a progressive deficit of retrograde axonal transport heralds the clinical expression of OPIDP [12].

Some of these biochemical aspects of OPIDP have been validated so far in man [13]. Here we report the distribution and some biochemical characteristics of NTE from several tissues in man as compared with those in the hen. For comparison the distribution of acetylcholinesterase (AChE) was also measured.

## *Experimental*

*Chemicals.* Di-isopropyl phosphorofluoridate (DFP) and phenylmethylsulphonyl fluoride (PMSF) were from Fluka AG Chem. Fabrik (Buchs, Switzerland). Diethyl *p*-nitrophenyl phosphate (paraoxon), which was purified according to Johnson [14], acetylthiocholine iodide and DTNB (5,5′-dithiobis-2-nitrobenzoic acid) were from Sigma Chem. Co. (St Louis, MO). A purified preparation of *N,N′*-di-isopropylphosphorodiamidic fluoride (Mipafox) and phenylsulphonyl fluoride (PSF) were a gift of Dr M. K. Johnson, MRC Laboratories (Carshalton, U.K.). Phenyl valerate (PV) was synthetized and purified according to Johnson [14].

*Buffers.* Tris buffers for NTE studies were as follows: 50 mM Tris/0.2 mM EDTA was adjusted (at 23°) to pH 8.0 with HCl or to pH 5.2 with 50 mM citric acid/0.2 mM EDTA; in the text these buffers will be identified by pH. For AChE assay, 100 mM phosphate buffer pH 7.4 at 23° was used.

*Tissue preparation.* Samples of human tissues were obtained from post-mortem examinations performed within 36 hr after death, during which time NTE and AChE activities are known to be stable [15]. The analyzed organs are listed in Table 1. Organs with evidence of gross pathology were discarded. Tissue samples were washed in cold Tris pH 8.0, dried and stored at −20° until assayed. Tissues were homogenized (10% w/v) in the same buffer with a Polytron homogenizer and then further diluted in Tris pH 8.0 or Phosphate buffer pH 7.4 for NTE and AChE assays, respectively.

*Identification and determination of NTE activity.* NTE is a PV-hydrolyzing esterase in the nervous tissue which is dissected from other esterases by using selective inhibitors [14]. Pre-incubation of tissue homogenates with paraoxon at pH 8.0, 37°, for 20 min, inhibits most of the PV esterase activity. If mipafox is also added in the incubation medium, more activity is inhibited. This additional decrease represents NTE activity. To define NTE activities in all tissues, we derived inhibition curves with paraoxon (0.1–1000 μM) of all PV esterases ("A" activity) in order to dissect activity resistant to paraoxon inhibition; PV esterases insensitive to paraoxon (40 μM) ("B" activity) were further titrated with mipafox (0.1–500 μM). The residual activity resistant to paraoxon and mipafox is called "C" activity. The reported NTE activities (B–C) were calculated according to Johnson [14] as follows: PV activity insensitive to paraoxon (40 μM) minus PV activity insensitive to both paraoxon (40 μM) and mipafox (50 μM).

NTE $I_{50}$s for mipafox, DFP and PMSF (pH 8.0, 37°, 20 min) were calculated as previously described [16].

*Determination of AChE activity.* AChE was measured in the organs where NTE activity was found, according to Ellman *et al.* [17].

*Aging of phosphorylated NTE.* Aging of DFP-inhibited NTE from human brain, liver and kidney, and hen brain was studied according to the procedure described by Clothier and Johnson [8]. Sample P, where paraoxon was substituted by PSF (250 μM), was prepared by incubating the 10% homogenate with PSF for 25 min at 37°, pH 8.0. The reaction was stopped by cooling at 0°, the mixture was centrifuged at 30,000 *g* for 20 min at 4°, the supernatant discarded and the pellet resuspended in Tris pH 5.2. The same procedure was adopted to prepare sample M where mipafox (200 μM) was added 5 min before cooling. The difference between the ability of P and M to hydrolyse PV represented NTE activity. P and M were incubated with DFP (20 μM at 37°, pH 5.2 for 2 min) and incubation was stopped by dilution (25-fold) with Tris pH 5.2, at 37°. Immediately and also 5 min after the dilution, aliquots of diluted samples were added to a KF (for reactivation) or KCl (for controls) solution (both 200 mM final concentration) at 37°. Reactions were stopped 10 min thereafter by cooling to 0°. After centrifugation at 30,000 *g* for 60 min at 4°, pellets were resuspended in Tris pH 8.0 and the substrate was added to measure NTE activity, as usual. Uninhibited samples from hen brain and human kidney were not significantly affected by the whole procedure. The recovery of NTE activity was somehow lower for human brain and liver (Table 2) and we don't know if this was due to partial solubility of NTE. Nevertheless for all organs the recovery of NTE activity was similar for KCl and KF-treated samples.

* Part of these results was communicated to the Second International Meeting on Cholinesterases, Fundamental and applied aspects, Bled, Yugoslavia, 17–21 September 1983.

† Abbreviations used: AChE, acetylcholinesterase; DFP, di-isopropyl phosphorofluoridate; NTE, Neuropathy Target Esterase; OP, organophosphorus esters; OPIDP, organophosphate-induced delayed polyneuropathy; PMSF, phenylmethylsulphonyl fluoride; PSF, phenylsulphonyl fluoride; PV, phenyl valerate.

Table 1. Phenyl valerate (PV) esterase and acetylcholinesterase (AChE) activities in human tissue

| Tissue | NTE (B–C)* (μmoles/m/g wet tissue) | NTE/B (%) (range) | AChE (μmoles/m/g wet tissue) |
|---|---|---|---|
| Adrenal | 1.31 ± 0.39 | 49–76 | 0.72 ± 0.39 |
| Bone marrow | 0.38 ± 0.23 | 55–88 | 0.88 ± 0.67 |
| Colon | 0.94 ± 0.70 | 64–78 | 0.46 ± 0.21 |
| Epididymis | 0.49 ± 0.09 | 65–71 | 0.62 ± 0.36 |
| Frontal cortex | 2.32 ± 0.21 | 75–76 | 34.78 ± 7.64† |
| Heart | 0.53 ± 0.09 | 61–69 | 0.39 ± 0.18 |
| Kidney | 2.11 ± 0.28 | 70–77 | 0.98 ± 0.80 |
| Liver | 2.71 ± 0.04 | 60–74 | 0.92 ± 0.30 |
| Lung | 0.51 ± 0.02 | 71–81 | 1.44 ± 0.93 |
| Ovarian duct | 0.12 ± 0.02 | 59–73 | 0.67 ± 0.49 |
| Ovary | 0.36 ± 0.15 | 64–70 | 0.67 ± 0.21 |
| Pituitary | 1.35 ± 0.51 | 68–73 | 0.75 ± 0.10 |
| Prostate | 0.51 ± 0.09 | 62–75 | 0.62 ± 0.21 |
| Red muscle | 0.25 ± 0.02 | 61–81 | 1.54 ± 0.72 |
| Spleen | 1.50 ± 0.12 | 48–55 | 1.18 ± 0.64 |
| Submandibular gland | 0.69 ± 0.19 | 71–88 | 1.78 ± 0.49 |
| Testis | 1.15 ± 0.46 | 67–74 | 0.46 ± 0.21 |
| Thyroid | 0.58 ± 0.11 | 72–84 | 0.36 ± 0.25 |

Tissues were sampled from three to five different bodies. NTE activity was not detected in choroid plexus, gall bladder and uterus where concentrations of paraoxon higher than 40 μM inhibited all the mipafox-sensitive PV-esterases. In duodenal wall, gall bladder, gastric wall and pancreas no B activity was measured. All assays were carried out in duplicate. Data are expressed as means ± SD. NTE activity is known to be present in blood lymphocytes (11.5 ± 2.5 nmoles/min/mg protein) [20, 21], platelets (13.3 ± 2.4 nmoles/min/mg protein) [21] and placenta (16.1 nmoles/min/mg protein) [22].

* B, Paraoxon-resistant PV-esterases (40 μM at 37°, pH 8.0, for 20 min); C, PV-esterases resistant to paraoxon and mipafox (40 μM and 50 μM, respectively at 37°, pH 8.0, for 20 min).

† Measured in nucleus caudatus.

Table 2. Aging of di-isopropylphosphorofluoridate inhibited NTE

| Tissue | % Recovery of NTE activity* | Reactivation by KF (%)† immediately after inhibition | 5 mins after inhibition |
|---|---|---|---|
| Grey matter (human) | 69, 57, 63 | 100, 62, 70 | 44, 35, 27 |
| Liver (human) | 56, 64 | 92, 42 | 18, 0 |
| Kidney (human) | 98, 88 | 42, 67 | 19, 45 |
| Whole brain (hen) | 95, 95 | 82, 61 | 37, 13 |

* Recovery was calculated in uninhibited samples undergoing the same 'reactivation' procedure.

† Reactivation by KF started either immediately after incubation with DFP or 5 min later. Percentages of inhibited NTE which is reactivated by KF were calculated from corresponding KCl-treated samples.

*Results and discussion*

Paraoxon (40 μM) inhibits 68–99% of PV-esterase activities in all organs. It is not clear whether this variability is due to different proportions or sensitivities of the mixtures. In the organs where the remaining PV-esterase activity was not significantly affected by paraoxon concentration higher than 40 μM (see Table 1), NTE was then identified with the titration of this remaining activity with mipafox. NTE $I_{50}$s for mipafox ranged from 12 to 22 μM, whereas $I_{50}$s for DFP and PMSF ranged from 0.52 and 0.62 μM and from 190 to 330 μM, respectively. These values were similar to those found in concurrent experiments with hen brain and spleen. DFP and mipafox values are also consistent with previously reported values for hen and human brain [16], hen spleen [18], and rooster testis [19]. PMSF $I_{50}$s are slightly higher than those previously reported [16], possibly because of a less active sample.

NTE activity is present in several organs in man and values are given in Table 1. The distribution of NTE among organs is different from that of AChE and also from that found in the hen. In concurrent hen experiments, NTE was assayed in organs where already assayed by others [18], NTE activity was detected in nervous tissue (brain 2.27 ± 0.06 μmoles/min/g wet tissue, N = 4) and spleen (1.01 ± 0.15 μmoles/min/g wet tissue, N = 3), but not kidney and liver; these results were similar to those already reported [16, 18]. Williams, however, reported some activity in hen liver [23]. NTE activity (7.39 ± 0.39 nmoles/min/mg of protein) is also demonstrated in rooster testis [19].

AChE activity was also found in several organs (Table 1) and the distribution was very different from that of NTE. AChE was reported to be present in human plasma, red blood cells, platelets, spleen, lymph nodes, red muscle and placenta [24, 25].

Table 2 compares the reactivation of diisopropyl phosphorylated NTE by KF (200 mM, 10 min, 37°, pH 5.2) at different times. KF was added immediately or 5 min after the end of incubation with DFP. Regardless of the tissue and species source of NTE, a decline over time in respon-

siveness of inhibited NTE to KF is clearly evident. The progressive loss of responsiveness to KF enables us to conclude that aging of DFP-inhibited NTE is a biochemical reaction common to human and hen NTEs.

In conclusion, NTE activity is widely present in human organs and some of its biochemical characteristics, like sensitivity to inhibitors and aging of the phosphorylated enzyme, are similar among different tissues and comparable to those of hen brain NTE.

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*Universita' degli Studi di Padova* ANGELO MORETTO*
*Istituto di Medicina del Lavoro* MARCELLO LOTTI
*Via Facciolati 71, I-35127,*
*Padova, Italy*

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* Author to whom correspondence should be sent.

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## Modification of keratin by the chemotherapeutic drug mitoxantrone

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A major focus of research on the mechanism of action of anthracycline and anthracene chemotherapeutic drugs has been on the interactions of these drugs with cellular DNA [1, 2]. Mitoxantrone is a member of a series of anthraquinones which was originally synthesized as a DNA-intercalating drug. This drug has proven to be an active agent in the treatment of human breast carcinoma, acute leukemia, malignant lymphomas, and hepatoma [3]. Studies on the mechanism of cytotoxic action of mitoxantrone have indicated that in intact cells the drug binds DNA by a non-