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AGE SENSITIVITY TO ORGANOPHOSPHATE-INDUCED DELAYED POLYNEUROPATHY

BIOCHEMICAL AND TOXICOLOGICAL STUDIES IN DEVELOPING CHICKS*

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Abstract—Young animals are resistant to organophosphate-induced delayed polyneuropathy (OPIDP). The putative target protein in the nervous system for initiation of OPIDP in the adult hen is an enzyme called Neuropathy Target Esterase (NTE), which is dissected by selective inhibitors among nervous tissue esterases hydrolysing phenyl valerate (PV). We report here that the pool of PV-esterases sensitive to paraoxon was different in peripheral nerves of chicks as compared to that of hens while that of brain and spinal cord was not. NTE activity decreased with age in brain, spinal cord and peripheral nerve, but its sensitivity to several inhibitors remained unchanged. In the adult hen more than 70% inhibition of peripheral nerve NTE by neuropathic OPs is followed by deficit of retrograde axonal transport, axonal degeneration and paralysis. Similar NTE inhibition in 40-day-old or younger chicks however is not followed by changes in retrograde axonal transport nor by OPIDP. Chicks aged 60 to 80 days are only marginally sensitive to a single dose of DFP otherwise clearly neuropathic to hens. *In vitro* and *in vivo* phosphorylation by DFP and subsequent aging of brain NTE is similar both in chicks and in hens. The recovery of NTE activity monitored *in vivo* after inhibition by DFP is faster (half-life of about 3 days) in chick peripheral nerves as compared to chick brain, hen brain and hen peripheral nerve (half-life of about 5 days). It is concluded that the reduced sensitivity to OPIDP in chicks is not due to differences in OP–NTE interactions. The resistance might be explained by a more efficient repair mechanism, as suggested by the faster recovery of peripheral nerve NTE activity.

Single doses of some organophosphorus (OP‡) esters cause organophosphate-induced delayed polyneuropathy (OPIDP) which is characterized by degeneration of long and large-diameter axons in spinal cord and peripheral nerves of sensitive species, including man [1–4]. The mechanism of OPIDP initiation is thought to be related to the inhibition of more than 70% of axonal Neuropathy Target Esterase (NTE) within hours of dosing, and to the following aging reaction. This reaction is a non-enzymatic change of phosphorylated NTE, which occurs rapidly and is characterized by the cleavage of a side chain, leaving a charged monosubstituted phosphoryl group attached to the protein. When aging has occurred, NTE activity is no longer reactivable by electrophiles such as fluoride. Progressive deficit of retrograde axonal transport then follows reaching the peak 7 days after treatment [5]. Ataxia and paralysis represent the clinical expression of axonal degeneration and appear within the following 3–7 days. At this time NTE activity is almost completely recovered [6]. Among several esterases of the nervous system which hydrolyse phenyl valerate (PV), NTE is dissected by means of selective inhibitors [1, 7]. NTE is defined as the PV-esterase activity which is resistant to relatively high concentrations of paraoxon (a non-neuropathic OP) and sensitive to mipafox (a neuropathic OP) [8]. The physiological substrate of NTE is unknown. NTE is also inhibited by compounds which do not cause OPIDP because the inhibited NTE they form cannot age [1]. When animals are pretreated with such compounds, they are protected from a subsequent challenging dose of a neuropathic OP [9]. Therefore the catalytic activity of NTE does not seem essential for the health of axons.

Several species are sensitive to OPIDP and the hen is the animal of choice for experimental OPIDP. It was reported that young animals of sensitive species including chicks do not develop OPIDP when treated with a dose of OP known to be effective in the adult [10, 11]. In such studies however, only brain biochemistry was performed in chicks of 49 days of age and no studies were reported on the relationship between NTE and other PV-esterases, the aging of phosphorylated NTE, and the recovery of NTE in the nervous tissue after inhibition at different ages. Measurements of axonal transport and histopathology were also not done.

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‡ Abbreviations: NTE, Neuropathy Target Esterase; OPIDP, organophosphate-induced delayed polyneuropathy; OP, organophosphates; DFP, diisopropyl-fluorophosphate; DBDCVP, di-*n*-butyl dichlorovinyl-phosphate; PV, phenylvalerate.

This paper reports the biochemical characteristics of the pools of PV-esterases in brain, spinal cord and peripheral nerves of chicks of different ages. The biochemical and physiological changes known to occur during OPIDP initiation have also been studied in chicks.

MATERIALS AND METHODS

Chemicals. Paraoxon (*O,O*-diethyl-*p*-nitrophenyl phosphate) (Sigma Chemical Co., St Louis, MO, U.S.A.) was purified according to Johnson [8]. Mipafox (*N,N*-diisopropylphosphorodiaminofluoridate) was purchased from Lark Enterprise (Webster MA, U.S.A.). Diisopropylfluorophosphate (DFP) was purchased from Fluka AG Chem Fab (Buchs, Switzerland). Di-*n*-butyldichlorovinylphosphate (DBDCVP) was a gift of RJ Richardson, University of Michigan (Ann Arbor, MI, U.S.A.). Phenyl valerate (PV), was synthesized and purified according to Johnson [8]. Physostigmine was purchased from the Sigma Chemical Co., and atropine sulphate from Ega-Chemie (Steinheim, F.R.G.). Purified tetanus toxin was kindly supplied by Dr K. Field, Albert Einstein College of Medicine, (Bronx, NY, U.S.A.), and iodinated with $Na^{125}I$ and chloramine-T as described by Miller and Spencer [12].

Animals and dosing. Ten-day-old chicks (White Leghorn) were obtained from a local breeder, fed *ad lib.*, and then dosed and killed at different ages. Adult hens (1.5–2.5 kg body wt, about 12 months of age) of the same strain were obtained from the same breeder. Animals were given DFP (1 or 1.3 mg/kg s.c.) or DBDCVP (1 mg/kg s.c.) or vehicle (glycerol formal, 0.1 mL/kg s.c.). To prevent DFP cholinergic toxicity animals were pretreated with atropine (20 mg/kg i.p.) and physostigmine (0.1 mg/kg i.p.). Chicks and adult hens were observed daily for ataxia from day 8 to day 20 and then twice a week. Clinical score was assessed according to a 0–4 point scale [11].

Tissues for biochemical assays. Animals were killed by decapitation; brain, lumbosacral spinal cord and sciatic nerves were immediately dissected and washed in ice-cool Tris/HCl buffer 50 mM, pH 8.0, containing EDTA 0.2 mM. Sciatic nerves were treated with modified Koenig buffer as previously described, to facilitate homogenization [6]. Tissues were cleaned, weighed and homogenized for immediate assay or kept frozen at −80° until used. Tissues were homogenized in Tris buffer and protein content measured according to Lowry *et al.* [13].

NTE activity. NTE activity in brain and spinal cord was measured according to Johnson [8]. NTE activity in peripheral nerves was measured according to Caroldi and Lotti [6] as further modified [14]. In some experiments paraoxon 256 μM was used, instead of the usual concentration of 40 μM, in order to ensure complete inhibition of paraoxon-sensitive PV-esterases (see results).

Titration of PV-esterases. The sensitivity of PV-esterases to paraoxon was assessed by incubating homogenates with increasing concentrations of paraoxon (0–1 mM final, 9–12 concentrations) for 20 min, 37°, pH 8.0, and the remaining activity was then assayed according to Johnson [8]. Mipafox titration curves were obtained by adding increasing mipafox concentrations (0–0.5 mM, 7–12 concentrations) to homogenates containing paraoxon (40 or 256 μM). The remaining activity was then assayed after 20 min incubation at 37°, pH 8.0 [8]. The sensitivity of NTE to inhibition by DFP or DBDCVP (I_{50}s) was assessed by adding to the homogenates containing paraoxon (40 or 256 μM) or paraoxon plus mipafox (50 μM) increasing concentrations of inhibitors as previously described [15].

Reactivation/aging of phosphorylated NTE. In vitro reactivation of DFP-inhibited NTE was studied according to the modified [16] method of Clothier and Johnson [17], where KF is used as reactivator. The aged inhibited NTE cannot be reactivated; KF would reactivate non-aged inhibited NTE, whereas KCl has no effect on either aged or non aged phosphorylated NTE. To determine the occurrence of *in vivo* aging of NTE, KF was added to samples of brain homogenates taken from animals which were treated 24 hr earlier with DFP (1 mg/kg s.c.). Control birds were treated with vehicle only. The inhibition of NTE measured after KF treatment was compared with that measured after KCl treatment.

Recovery of NTE activity. Twenty-four-day-old chicks were treated with DFP (1.3 mg/kg s.c.) or vehicle; hens were treated with DFP (0.9 mg/kg s.c.) or vehicle. Animals were killed at intervals and NTE activity was measured in brain and peripheral nerve.

Retrograde axonal transport. Twenty-one-day-old chicks were treated with DBDCVP (1 mg/kg s.c.). In the adult hen this dose causes OPIDP and 65–75% decrease of retrograde axonal transport on day 7 after treatment [5]. Retrograde axonal transport was measured as previously described [5]. Due to the short sciatic nerves of chicks the peak of radiolabel accumulation in dorsal root ganglia and ventral spinal cord was observed 24 hr after injection. Consequently, to study retrograde axonal transport in chicks, we chose a time-point at 20 hr.

Histopathology. At 7, 14 and 21 days after DBDCVP treatment (1 mg/kg s.c.) animals were perfused as previously described [5]. Cervical, thoracic and lumbo-sacral spinal cord and sciatic nerve were dissected, processed and examined for axonal degeneration by bright-field microscopy.

RESULTS

Titration of PV-esterases

Titration curves of brain and spinal cord PV-esterases with paraoxon and with mipafox plus paraoxon (40 μM) were similar both in chicks and adult hens (data not shown). In peripheral nerve, on the contrary, the paraoxon titration curves, changed with age (Fig. 1.) Inspection of the figure reveals that PV-esterases activity of 20-day-old chicks (Fig. 1a) was substantially inhibited by preincubation with 10^{-5} M paraoxon (or, higher, 4×10^{-5} M), but the inhibition was markedly greater at 10^{-4} and again at 10^{-3}. In the adult hen (Fig. 1d) the increase from 10^{-4} M to 10^{-3} M paraoxon caused smaller change. This difference disappeared with the increase of age and at 70 days of age the curve was similar to that of the adult. It cannot be ascertained whether

Table 1. NTE activity in the nervous tissues of chicks at different ages

Age (days)	Brain		Spinal cord		Peripheral nerve	
	NTE	$\frac{B-C}{B}$ %	NTE	$\frac{B-C}{B}$ %	NTE	$\frac{B-C}{B}$ %
11	39 ± 1	76 ± 1	17 ± 1	67 ± 2	ND	ND
20	38 ± 6	77 ± 1	13 ± 1	61 ± 4	2.5 ± 0.1	31 ± 2
29	40 ± 7	76 ± 1	12 ± 1	61 ± 1	2.1 ± 0.4	32 ± 3
41	33 ± 1	75 ± 1	11 ± 1	57 ± 1	1.8 ± 0.4	37 ± 5
50	30 ± 1	75 ± 1	10 ± 1	56 ± 2	1.6 ± 0.4	35 ± 3
60	31 ± 1	77 ± 1	10 ± 1	57 ± 2	1.4 ± 0.2	39 ± 4
70	31 ± 1	76 ± 1	9 ± 1	53 ± 1	1.7 ± 0.4	38 ± 3
>365	26 ± 1	77 ± 1	5 ± 1	48 ± 3	1.0 ± 0.1	38 ± 8

NTE activity is given as specific activity (nmol/min/mg of protein) and as the percentage of paraoxon resistant PV-esterases (mean ± SD, N = 3). B = PV-esterase activity resistant to paraoxon (256 μM). C = PV-esterases resistant to both paraoxon (256 μM) and mipafox (50 μM). NTE = B − C. ND: not done because of insufficient tissue.

this represents a change in the sensitivity of PV-esterases. In order to ensure complete inhibition of paraoxon-sensitive PV-esterases in the sciatic nerve of chicks, the paraoxon concentration was increased to 256 μM when NTE activities were calculated (Table 1). The PV-esterase activity removed from 20-day-old chick nerve by increasing paraoxon concentration to 256 μM was numerically greater than that achieved by adding mipafox to paraoxon (40 μM).

Figure 1 together with the NTE I_{50} data for mipafox reported in Table 2 shows that no changes in mipafox sensitivity of paraoxon resistant PV-esterases occurred during development.

NTE activity

Table 1 reports the values of NTE activity in the nervous system of chicks of different ages. In all tissues NTE activity decreased with the increase of age. The ratio NTE/paraoxon resistant PV-esterases decreased in the spinal cord, slightly increased in the peripheral nerve and remained unchanged in the brain.

When NTE I_{50}s were calculated for mipafox, DFP and DBDCVP in brain and peripheral nerves, no age-related differences were detected (Table 2). The different NTE I_{50}s of DBDCVP according to the nervous tissue source are known [14]. When phosphorylated by DFP both *in vitro* (Table 3) and *in vivo* (see below), NTE underwent the aging reaction both in the hen and the chick because reactivation by KF was not possible.

Inhibition, aging and recovery of NTE in vivo

Twenty-four hours after a single dose of DFP, NTE was inhibited throughout nervous system both in chicks and in hens (Table 4). A somewhat lower, but still higher than threshold inhibition was found in 21-day-old chicks. This might be related to a quicker disposal of the compound in chicks of this age. Diisopropylphosphorylated NTE was not reactivable by KF (i.e. has aged) in the brain of both chicks (21 days of age) and hens when treated with DFP (1 mg/kg s.c.), 24 hr earlier (N = 3). Figure 2 shows the time-course of reappearance of NTE activity in brain and peripheral nerves of 24-day-old chicks when treated with DFP. The reappearance of NTE activity was faster in peripheral nerves than in the brain. The half-lives were calculated from the best fitted line with regression analysis of experimental points and found to be 4.7 days in the brain ($r = 0.98$) and 2.8 days in peripheral nerves ($r = 0.96$). Similarly, half-lives were also calculated in brain and peripheral nerve of adult hens treated with DFP and found to be 5.2 and 4.7 days, respectively (data not shown), which are similar to those already published [6, 18].

Retrograde axonal transport

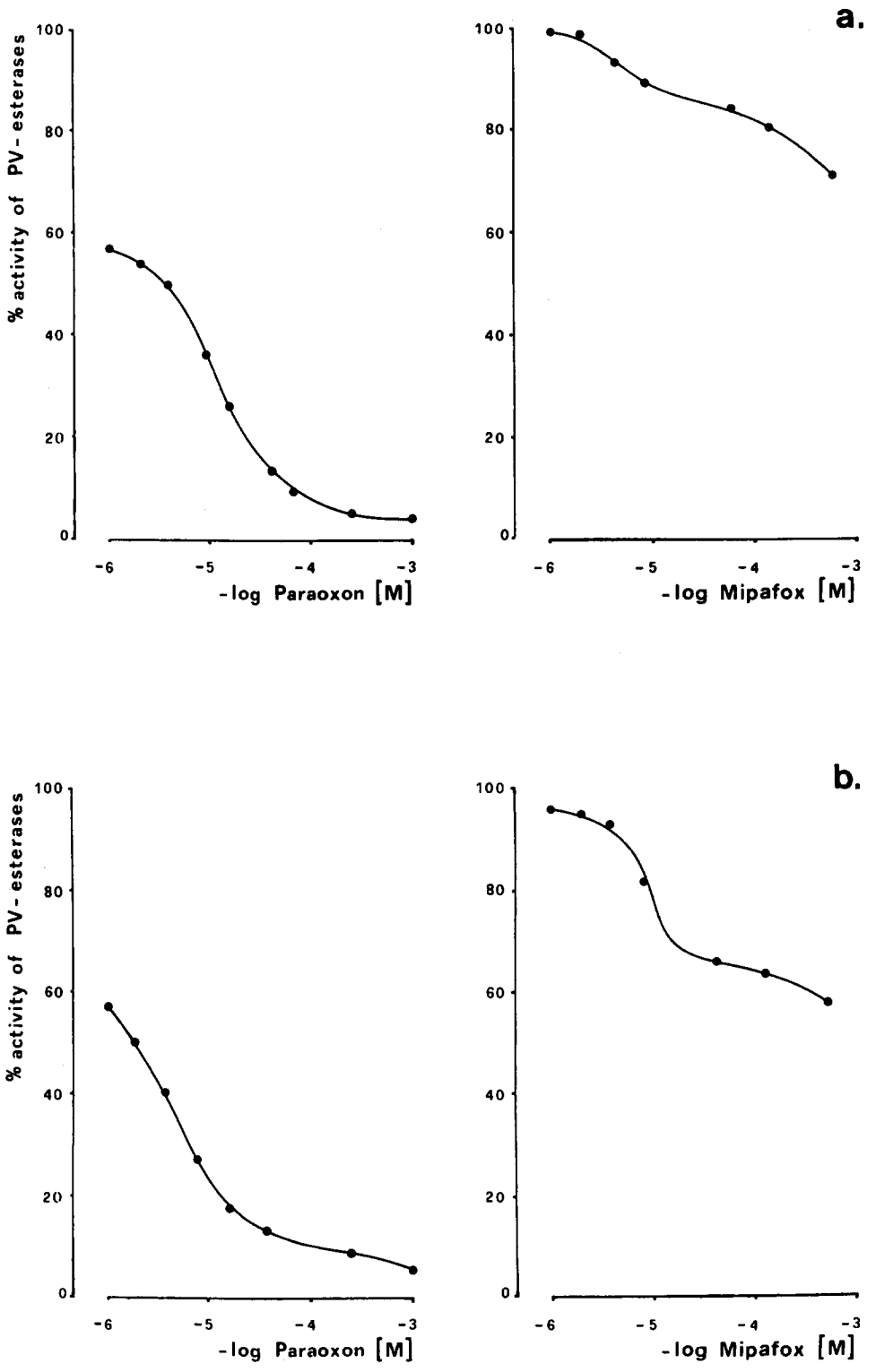
In chicks, NTE inhibition above the threshold was not related to significant decreases in the amount of retrogradely transported [I^{125}]tetanus toxin as shown in Table 5. On the contrary, high NTE inhibition in peripheral nerve of hens correlated with reduction of retrograde axonal transport and OPIDP.

Expression of OPIDP

Tables 4 and 5 show that sensitivity to OPIDP after single doses of DFP and DBDCVP developed with age. OPIDP does not develop in chicks treated with neuropathic OPs at the age of 40 days or younger, whereas 60–80-day-old chicks developed mild and partially reversible ataxia. Similarly treated hens developed severe and irreversible signs of OPIDP.

DISCUSSION

This study confirms that chicks up to 6–7 weeks of age are resistant to OPIDP as caused by single doses of OPs and when this toxicity is assessed clinically and histopathologically [11, 19]. Changes on NTE biochemistry, are unlikely to be the cause of chicks resistance. NTE activity seems to have similar characteristics in the hen and in developing chicks, when its sensitivity to inhibitors and the aging of phosphorylated NTE, both *in vitro* and *in vivo* were evaluated comparatively. Differences however have been detected between young chicks and hens and some parallel the development of sensitivity to OPIDP. NTE activity decreases from 11 days of age until adulthood and this is particularly evident in the spinal cord and peripheral nerves which are the target organs for OPIDP. The decrease of NTE


a.
% activity of PV-esterases
100
80
60
40
20
0
-6
-5
-4
-3
-log Paraoxon [M]
-log Mipafox [M]
b.
% activity of PV-esterases
-log Paraoxon [M]
-log Mipafox [M]

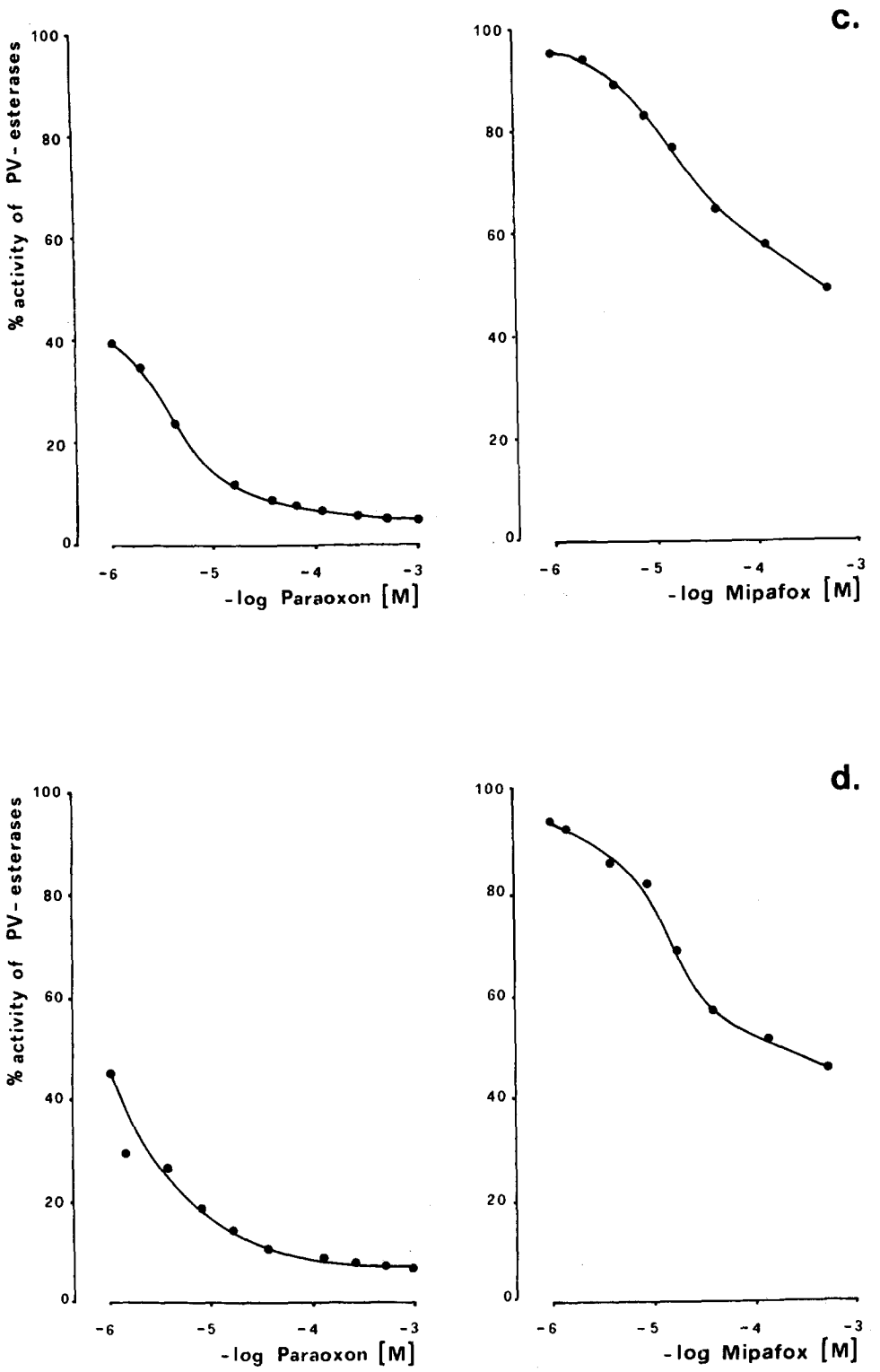


Fig. 1. Age-sensitivity of peripheral nerve PV-esterases to inhibitors in chicks of (a) 20, (b) 40, (c) 70 days of age and (d) in the adult hen. On the left hand side the titration curves with paraoxon. 100% of total PV esterase were 4.4, 4.0, 4.0, 3.8 μmol/min/g of tissue respectively. PV-esterases resistant to paraoxon (40 μM) have been then titrated with mipafox (right hand side). 100% of such PV-esterase were 0.59, 0.53, 0.37, 0.39 μmol/min/g of tissue, respectively.

Table 2. *In vitro* sensitivity (I_{50}) of NTE from chicks and hen nervous tissues to organophosphate inhibition

Age (days)	Brain I_{50} Mipafox (μM)	Brain I_{50} DFP (μM)	Brain I_{50} DBDCVP (nM)	Peripheral nerve I_{50} Mipafox (μM)	Peripheral nerve I_{50} DFP (μM)	Peripheral nerve I_{50} DBDCVP (nM)
11	8.0	0.9	ND	12.5	0.7	ND
20	8.0	1.0	3.2	(13.3)	(0.9)	(12.2)
>365	8.5	0.8	2.5*	9.5	0.7	8.0*
				(13.3)	(1.0)	(14.0)

NTE was inhibited at 37°, pH 8.0 for 20 min and I_{50}s were then calculated as described in methods. Data in brackets were obtained measuring NTE activity with paraoxon 256 μM.
* From Moretto *et al.* [14]. ND = not done.

Table 3. Reactivation by KF of brain NTE inhibited *in vitro* by DFP in 21–23-day-old chicks and in hens

		NTE Inhibition (2 mM, 2 min, pH 8.0, 37°) Time 0 min* % activity of the inhibited sample treated with KCl (a)	KF (b)	Time 8 min† % activity of the inhibited sample treated with KCl (c)	KF (d)	Reactivation‡ (e)	(f)
Chick	(N = 4)	10 ± 2	49 ± 1	10 ± 3	17 ± 2	44 ± 9	8 ± 1
Hen	(N = 3)	7 ± 3	37 ± 2	7 ± 3	16 ± 2	33 ± 3	10 ± 1

* Per cent of activity was calculated from non-inhibited samples. The recovery of activity of non-inhibited samples undergoing the entire procedure was about 85%. KCl and KF treatments (10 min, 37°, pH 5.2) started immediately after the end of DFP incubation.
† The same KCl and KF treatments started 8 min after the end of DFP incubation while samples were at 37°, pH 8.0 to allow aging to take place.
‡ Reactivation was calculated as follows:

$$e = \frac{b - a}{100 - a} \times 100 \quad f = \frac{d - c}{100 - c} \times 100.$$

Data are expressed as means ± SD.

Table 4. Biochemical and clinical effects of a single dose of DFP (1 mg/kg s.c.) in chicks and hens

Age (days)	% NTE inhibition* Brain	Spinal cord	Peripheral nerve	Clinical score† Day 14	Day 30
21	72 ± 6	73 ± 8	79 ± 6	0,0,0,0,0	0,0,0,0,0
42	84 ± 1	77 ± 2	90 ± 9	0,0,0,0,0	0,0,0,0,0
61	80 ± 8	77 ± 4	84 ± 3	1.1 ± 0.6	0.5 ± 0.4
82	92 ± 5	80 ± 1	84 ± 7	1.3 ± 0.6	0.7 ± 0.6
>365	88 ± 6	87 ± 3	93 ± 1	4,4,4,4,4	ND

* Calculated from the mean activity of same age animals treated on the same day with vehicle only (mean ± SD, N = 3–4).
† Score of individual animals or mean ± SD (N = 5).
ND: not detectable. Maximal clinical score was reached on day 22 when animals were killed because they were unable to eat.

activity however is continuing beyond the period of absolute resistance, suggesting that the amount of NTE would be unlikely related to OPIDP insensitivity.

Increasing age is paralleled by changes in the titration curves of peripheral nerve PV-esterases with paraoxon. From these data however it cannot be ascertained whether these age-related changes reflect a higher sensitivity of such esterases to the inhibitor.

It was proposed that the insensitivity of chicks to single doses of OPs should be attributed to the rapid metabolism and excretion of these compounds in young birds [20, 21]. Our results together with similar published data [11] do not support this hypothesis, because NTE inhibition achieved with single doses of OPs are at any age well above the threshold for OPIDP initiation and yet different clinical responses have been observed in chicks. OPIDP is correlated

Table 5. Effects of DBDCVP (1 mg/kg s.c.) on retrograde axonal transport, histopathology and clinical behaviour

Age* (days)	Peripheral nerve NTE (% control)†	Retrograde axonal transport (% control)‡ Motor axons	Retrograde axonal transport (% control)‡ Sensory axons	Axonal degeneration§	Clinical score‖
21	19 ± 4	78 ± 22¶	103 ± 42¶	—	0,0,0,0,0,0
>365**	17 ± 6	35 ± 9††	26 ± 19††	+	3.6 ± 1.0

* On the day of treatment with DBDCVP.

† Measured 24 hr after treatment (mean ± SD, N = 4) and calculated from the mean value of four control animals.

‡ Assessed on day 7 after treatment.

§ Assessed on days 7, 14 and 21 after treatment, in three animals per time point. Histopathological changes were observed on days 14 and 21 in hens.

‖ Assessed on day 15 after treatment. Score of individual animals or mean ± SD (N = 6).

¶ Not significantly different from controls, Student's *t*-test (mean ± SD, N = 9).

** Taken from Moretto *et al.* [14].

†† Significantly different from controls (mean ± SD, N = 12).

with NTE phosphorylation above the threshold in peripheral nerve axons and not in the brain [22, 23]. Some OPs might selectively affect brain and not spinal cord and peripheral nerve NTE, possibly because of different access [24]. Peripheral nerve NTE might also display a different sensitivity to inhibitors [14]. Our comparative data on NTE inhibition showed that NTE inhibition was similar in all parts of the nervous system.

The speed of NTE reappearance in the peripheral nerve of chicks was much faster than that in the brain and this difference was not detected in the adult hen. However in the brain of both chicks [11] and hen [6, 18] the speed is similar. Because repairing capabilities of developing nervous system are more efficient than that of the adult, a faster replacement of newly synthesized NTE might be related to the resistance to OPIDP displayed by chicks. For instance, when axons are regenerating, specific changes occur in the pattern of fast transported proteins [25]. Since NTE travels along nerves at a speed corresponding to fast anterograde transport

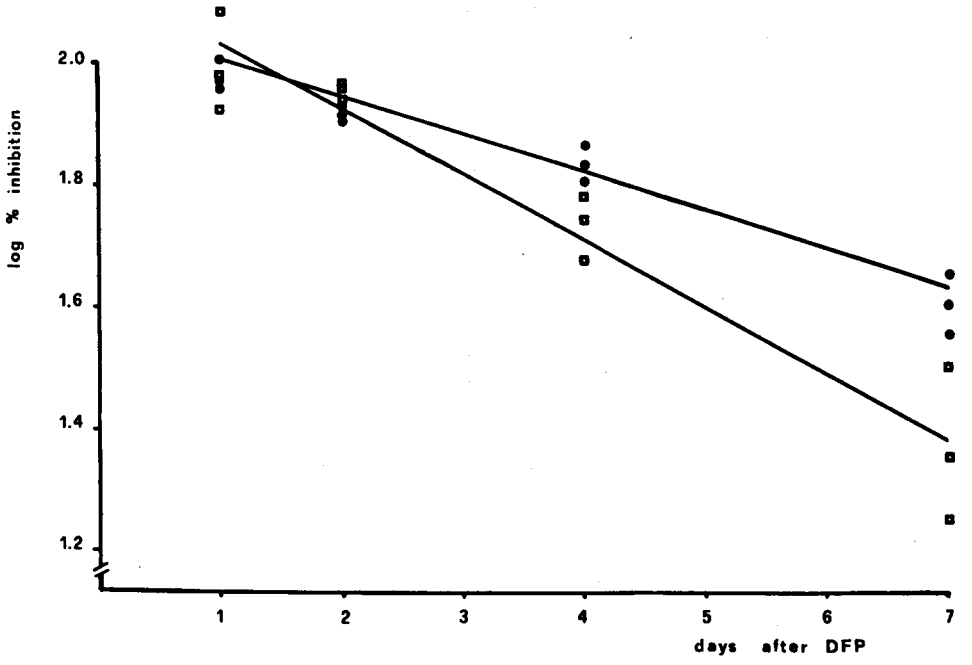


Fig. 2. Time-course of NTE reappearance in brain (●) and peripheral nerve (□) of chicks dosed with DFP (1.3 mg/kg s.c.). 100% inhibition corresponded to 85% in brain and to 69% in peripheral nerve of that measured in concurrent control birds (N = 6, mean NTE activity ± SD was 25 ± 4 and 1.6 ± 0.3 nmol/min/mg of protein in brain and peripheral nerve, respectively) 24 hr after dosing. Each point represents the result from one animal at a given time. The calculated regression lines are shown. Peripheral nerve is significantly different from brain ($P < 0.01$).

[26], our findings could be interpreted as the result of a more efficient repair. The higher speed of NTE reappearance might be non-specific or suggesting that NTE activity itself is directly involved in the repairing mechanisms. A marked reduction of retrograde axonal transport was shown to be a primary and selective effect of OPs during the pathogenesis of OPIDP [5]. Retrograde axonal transport was not affected in chicks treated at 21 days of age, suggesting that the observed NTE modifications are not followed by the physiological changes known to lead to OPIDP.

In conclusion the age-related resistance to OPIDP cannot be explained either with differences in the pharmacokinetics of neuropathic OPs nor in the toxin-target interaction as it is presently known. Differences in the pool of PV-esterases between the developing peripheral nerve and that of the adult hen have been shown, and the faster reappearance of NTE in the peripheral nerve may be related to some unknown repair mechanisms of the initial biochemical lesion.

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