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The Effect of Trichlorfon and other Organophosphates on Prenatal Brain Development in the Guinea Pig*

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The organophosphates trichlorfon, dichlorvos, dimethoate, soman, triortho-cresyl phosphate (TOCP), and the diethoxy-analogue of trichlorfon (O,O-diethyl 2,2,2-trichloro-1-hydroxyethylphosphonate, ethyl-trichlorfon), were administrated to guinea pigs between day 42 and 46 of gestation. When the offsprings were examined at birth, there was a severe reduction in brain weight in the case of trichlorfon and dichlorvos, but not after treatment with the other organophosphates. The reduction in weight was most pronounced for cerebellum, medulla oblongata, thalamus/hypothalamus and quadrigemina. The effect was less marked for cerebral cortex and hippocampus. Since soman, a potent anticholinesterase, and TOCP, an inhibitor of neuropathy target esterase, did not show any effects, this excludes that the brain hypoplasia can be caused by inhibition of these two enzymes. Further, the lack of effect with ethyl-trichlorfon has shed some light on the part of the trichlorfon molecule which could be involved in the formation of the hypoplasia. It is suggested that alkylation of DNA may be involved in the development of the lesion. The possible consequences for a teratogenic effect of trichlorfon and dichlorvos on humans are discussed.

KEY WORDS: Brain development; dichlorvos; dimethoate; guinea pig; organophosphates; soman; teratogen; trichlorfon, tri-o-cresyl phosphate; Acetylcholinesterase.

INTRODUCTION

Trichlorfon is an organophosphonate widely used in veterinary medicine against endo- and ectoparasites and in aquaculture industry also for controlling salmon lice. In human medicine it is used against the trematode *Schistosomiasis haematobium*. The teratogenic potency of trichlorfon was first discovered in pigs when the piglet offspring of sows treated late in gestation showed severe locomotional disturbances including ataxia that were related to a reduction in the cerebellar weight (1–7).

The lesions were later reproduced in guinea pigs, but not in rats (8). Dose-response studies have shown that 100 mg/kg of trichlorfon given to guinea pigs on 3 consequtive days during days 40–50 of gestation results in offspring with brain hypoplasia, ataxia and tremor. The effect is particularly marked in cerebellum, but there is also a distinct reduction in total brain weight. There seems to be only minor effects before or after this period (9). Also no effects have been described to other parts of the body.

The finding of brain lesion produced in pigs and guinea pigs raises two important questions. First, can the lesion produced by trichlorfon provide a better understanding of the toxic effect of chemicals on brain cells during early development? Second, does the widespread use of trichlorfon and related compounds constitute a risk for teratogenic effects in humans? A teratogenic effect of trichlorfon in humans was recently indicated in an epidemiological study among children of Hungarian

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women, who had been exposed to high concentration of trichlorfon during pregnancy, due to the consumption of fish heavily contaminated by trichlorfon (10).

In the present communication we have elucidated the mechanism for the teratogenic effect of trichlorfon by using different chemical agents which cover some of its chemical and pharmacological properties. The inhibition of acetyl cholinesterase observed after administration of trichlorfon is due to its metabolite dichlorvos (11), and it is an open question whether dichlorvos is involved in the brain lesion. Dichlorvos itself is a well established insecticide. Ethyl-trichlorfon has similiar chemical structure to trichlorfon except for a substitution of the two methoxy-groups with ethoxy-groups and produces metabolites similar to trichlorfon. Compared to trichlofon, soman is a more potent inhibitor of acetylholinesterase, and TOCP is a more established inhibitor of neuropathy target esterase than trichlorfon. The effects of soman, TOCP, dichlorvos and ethyl- trichlorfon on development of brain have not previously been investigated under similar conditions.

EXPERIMENTAL PROCEDURE

Animals. We used conventional outbred albino guinea-pigs (Ssc:AL, Mol:DHF from Statens Seruminstitutt, Hvidsten avlsgård, Denmark). The pregnant dams were kept individually in macrolon cages with metal bar covers. They were fed a commercial pelleted diet (Ewos, Södertalje, Sweden, and T. Skretting A/S, Stavanger, Norway) and tap water added ascorbic acid (70-100 mg/ml) ad libitum. There was a 12:12 hours light:dark cycle. Room temperature was 22°C and relative humidity was 40–60%.

The day of conception was determined by using a post partum mating procedure. Guinea pigs have a new oestrus within 24 hours after the (proceeding) delivery. The male was present immediately after birth, and the day of conception of the pregnancy was set to the day of birth of the last litter. The animals were born between day 69 and 72 of gestation.

Organophosphates. Dichlorvos (99% pure) and dimethoate (99% pure) was obtained from Riedel-de Haën. Trichlorfon (97% pure) was obtained as Neguvon® from Bayer Chemie AG. Triortho-cresyl phosphate (TOCP) (90–95% pure) was obtained from K & K Labs. Soman (1,2,2 trimethyl propyl methylphosphonofluoridate) (97% pure) and the ethyl analogue of trichlorphon (diethyl-(2,2,2-trichloro-1-hydroxyethyl)-phosphonate) (>95% pure), here called ethyl-trichlorfon, were syntesized at Norwegian Defense Research Establishment. Ethyl-trichlorfon was prepared by mixing chloral with diethyl hydrogen phosphite (12). Soman was prepared by mixing 1,2,2 trimethylpropanol with a mixture of methyl phosphonyl dichloride and methylphosphonyldifluoride (13). The purity of trichlorfon, soman and ethyl-trichlorfon was determined with nuclear magnetic resonance (NMR).

Experimental Design. Permission to carry out the experiments were given by the animal-use ethical committee of Oslo University. Since the agents tested are very toxic, we have chosen to give different doses to different litters. There was usually one litter for each dose of an organophosphate except for trichlorfon and for the control of which we had many litters. For the dose of TOCP and dimethoate we had two litters with the same dose. The days for administration and doses are given in Table I. TOCP was given early because it has to be metabolized to the cyclic substance o-tolyl saligenin phosphate to be able to inhibit neuropathy target esterase (14). 15 mg/kg of dichlorvos was the largest dose that could be received by the pregnant dams without the development of cholinergic symptoms. The turnover of dichlorvos in the body is rapid (15) and to have a more continuous treatment, we also administered 15 mg/kg dichlorvos with 12h intervals (expressed as 15 × 2). We gave 7 μg/kg of soman since a dose of 13 μg/kg which is well accepted by female guinea-pigs for 11 days (16), was highly toxic to pregnant animals.

The pups were weighed and decapitated within 24h after natural delivery. The brains (without the olfactory bulbs) were placed on ice and the following regions were dissected; medulla oblongata, cerebellum, quadrigemina (superior and inferior colliculi), hippocampus, cerebral cortex and diencephalon (thalamus and hypothalamus).

Biochemical Analysis. The brain regions were homogenized in 0.32 M-sucrose and choline acetyltransferase (17), glutamate decarboxylase (18) and acetyl cholinesterase (19) were assayed by previously described methods. The specific activity is expressed as μmol substrate catalysed per gram protein and hour.

RESULTS

In Fig. 1 is shown the marked effect of trichlorfon on the brain size of the newly born guinea pig. It can be seen that cerebellum was severly reduced in size, but also medulla-ponds and hypothalamus with the mammillary bodies were reduced.

There were no significant differences in body weight between the organophosphate treated pups and the control pups (Wilcoxon, $p > 0.05$, Table I). The reduction in brain weights was not a function of a reduction in body weights. Trichlorfon treatment resulted in significant reduction in the weight of the total brain (on average 29% reduction) and of most brain regions. The reduction in the weight of different brain regions were 77% in the cerebellum, 54% in the medulla oblongata, 45% in the quadrigemina, 36% in diencephalon, and 11% in the cerebral cortex. The hippocampus was unaffected. Administration of trichlorfon gave a 64% reduction in red blood cell acetyl cholinesterase after 1 hour, but the activity was completely reversed within 24 hours.

Of the other organophosphates tested, only treatment with dichlorvos clearly showed the same effects as trichlorfon. In this case there was also a dose-response relationship. A dose of 15 mg/kg dichlorvos every 12 hours gave a significant reduction in the total weight of the brain and the most severe reduction was, as was also the case for trichlorfon, in the cerebellum, medulla oblongata, diencephalon, and quadrigemina. The litter receiving dichlorvos every 24 hours only, showed no significant reduction in brain weight. On a dose basis

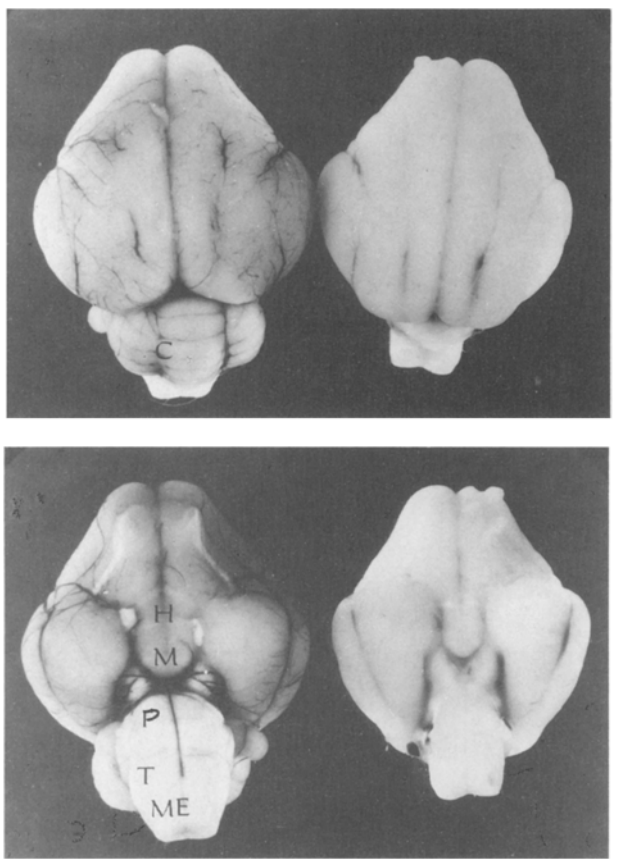

Fig. 1. The figure shows the brains of a central (left) and trichlorfon (right) treated rats. The top figure shows the dorsal view and the bottom the ventral view. Major reductions can be seen in cerebellum (c), hypothalamus (H), corpus mammilare (M), pons (P), corpus trapezoideum (T), and medulla oblongata (ME).

dichlorvos is much more efficient in producing lesions than trichlorfon. Since the mother expressed slight symptomes with the dichlorvos dosage regime, we did not increase the dose.

Treatment with ethyl-trichlorfon in doses comparable to trichlorfon, gave only a small reduction in brain weight. With the highest doses of ethyl-trichlorfon there was a small reduction in total brain weight and the reduction was significant also in medulla oblongata and quadrigemina, but not in cerebellum. In the second litter given 125 mg/kg, there was also a reduction in these two regions, but it did not reach significance. The inhibition of acetyl cholinesterase caused by 125 mg/kg of the diethyl analog of trichlorfon lasted much longer than for trichlorfon and the cholinesterase activity was reduced to 51% of control even after 24 hours. The toxicity of ethyl-trichlorfon was higher than that of trichlorfon

Table I. The Effect of Different Organophoshates on the Total Brain Weight and the Weight of Different Brain Regions in New Born Guinea Pigs

Substance	Dose/ Day mg/kg/ Day	Day of Administration	Body Weight, g	Total Brain Weight, g	Cerebellum, mg	Medulla, mg	Diencephalon, mg	Hippocampus, mg	Quadrigemina mg	Cortex mg
Saline	9	42,43,44,45	87 ± 21 (19)	2,62 ± 0,16 (15)	287 ± 29 (9)	189 ± 17 (9)	302 ± 31 (8)	179 ± 25 (9)	108 ± 24 (9)	1308 ± 123 (9)
Trichlorfon	125	42,43,44	82 ± 27 (10)	1,86* ± 0,22 (9)	67* ± 29 (6)	86* ± 23 (5)	193* ± 29 (6)	188 ± 29 (5)	59* ± 9 (5)	1170* ± 129 (6)
Dichlorvos	15	42,43,44	88 ± 12 (4)	2,47 ± 0,19 (3)	293 ± 54 (3)	178 ± 52 (3)	245* ± 40 (3)	142* ± 8 (3)	—	1312 ± 140 (3)
Dichlorvos	15 × 2	42,43,44	99 ± 16 (4)	2,25* ± 0,04 (4)	185* ± 20 (4)	142* ± 8 (4)	217* ± 9 (4)	212 ± 4 (4)	83* ± 7 (4)	1351 ± 41 (4)
Dichlorvos	15 × 2	44,45,46	99 ± 10 (4)	2,31* ± 0,04 (4)	237* ± 15 (4)	142* ± 4 (3)	248* ± 11 (3)	202 ± 6 (3)	85* ± 8 (4)	1320 ± 45 (4)
Ethyl Trichlorfon	125	44,45,46	105 ± 6 (5)	2,81 ± 0,04 (5)	319 ± 12 (4)	186 ± 10 (4)	366 ± 22 (4)	233 ± 13 (4)	98 ± 29 (4)	1508 ± 35 (4)
Ethyl Trichlorfon	138,121	42,44	106 ± 2 (2)	2,47* ± 0,08 (3)	286 ± 9 (3)	163* ± 1 (3)	318 ± 21 (3)	192 ± 16 (3)	73* ± 9 (3)	1352 ± 48 (3)
Soman	7,7,3 **	42,43,44	81 ± 15 (6)	2,53 ± 0,16 (6)	278 ± 18 (5)	161 ± 12 (5)	381 ± 60 (5)	162 ± 19 (5)	86 ± 12 (5)	1367 ± 99 (5)
Soman	7,7,5 **	42,43,44	100 ± 11 (3)	2,84 ± 0,08 (3)	318 (1)	187 (1)	390 (1)	154 (5)	90 (1)	1548 (1)
TOCP	100	41,43	90 ± 26 (9)	2,61 ± 0,15 (9)	307 ± 26 (2)	208 ± 17 (2)	342 ± 29 (2)	190 ± 30 (2)	116 ± 14 (2)	1546 ± 9 (2)
Dimetoate	72,90,90	40,41,42	112 ± 5 (4)	2,76 ± 0,05 (4)	307 ± 18 (3)	178 ± 2 (3)	321 ± 33 (3)	232 ± 13 (3)	108 ± 3 (3)	1353 ± 44 (3)

The mothers were treated at indicated days of gestation. The results are expressed as mean value ± SD (number of off-spring)
* $p \leq 0.05$
** µg/kg/day

itself (300 mg versus 450 mg i.p., own observations) and this restricted its use in the experiments. With the highest dose of ethyl-trichlorfon, we were not able to add ethyl-trichlorfon within 24 hours after the first administration.

Administration of soman was not accompanied by any reduction in brain weights. Twenty four hours after the first administration of the irreversible acetyl cholinersterase inhibitor soman, the esterase activities were 37% and 26% of control in the blood of the two dams respectively, and 24 hours after the second administration the activities were 41% and 39% of control. The toxic dose had to be reduced on the 3rd day to avoid toxic symptomes of the mother.

To our surprise, the large reductions in brain weight from the exposure to different agents were not accompanied by any specific changes in transmitter enzymes of any brain region examined. The reduction therefore seemed to be rather general and comprised all types of neurons rather than affecting a specific type e.g cholinergic neurons (Table II).

DISCUSSION

In the present experiments we have found a reduction in brain weight during development in guinea pig of trichlorfon and dichlorvos, but no or minor effects of ethyl-trichlorfon, soman and TOCP. Previously it was suggested that the effect of trichlorfon could be due to the inhibition of acetyl cholinesterase (8). The enzyme is extremely high in placenta and may possibly play a role in nutrient uptake across the placenta barrier (20). This does not seem to be the case. Soman, that is a specific irreversible inhibitor of this enzyme (21), did not produce effects like those of trichlorfon, on guinea-pig brain, even if the acetyl cholinesterase inhibition was more pronounced and longer lasting. Likewise, ethyl-trichlorfon, which also produces a stronger and a longer lasting inhibition of acetyl cholinesterase than trichlorfon, did not have severe effects on guinea pig brain. Atropin given together with trichlorfon or shortly afterwards did not prevent the teratogenic effect of trichlorfon (data not shown).

Trichlorfon is a potential inhibitor of neuropathy target esterase via dichlorvos, and one might expect that inhibition of this enzyme could play a role in the development of the brain hypoplasia. There were, however, no effects on brain weights of TOCP which is a good inhibitor of neuropathy target esterase (22). It therefore does not seem to be via this route that trichlorfon acts as a teratogen. In agreement, ethyl-trichlorfon that would be an even better inhibitor of neuropathy target esterase via ethyl-dichlorvos (23), did not give a severe brain hypoplasia.

Besides its phosphorylating abilities dichlorvos and to a lesser extent trichlorfon can methylate DNA and other cell structures (24–27). Dichlorvos is more than 10 times more effective than its ethyl-analog in alkylating DNA (28). Similar differences is expected between trichlorfon and ethyl-trichlorfon. It is therefore of interest that dichlorvos and trichlorfon but not ethyl-trichlorfon produced severe brain hypoplasia. Wooder and Wright (29) did not think that high exposures of dichlorvos were a methylating hazard to mammalian DNA in vivo. Dimethoate has also methoxy groups and can methylate DNA to nearly the same extent as dichlorvos in in vitro-tests with *Escherichia coli* and *Saccharomyces cerevis-*

Table II. The Effect of Organofosfate Treatment on Transmittor Enzymes in Brain Regions of New Born Guinea Pig

	Agent	Cerebellum	Medulla	Cerebral Cortex
Glutamate decarboxylase	Saline	91 ± 21 (4)	102 ± 9 (4)	115 ± 15 (4)
	Trichlorfon	82 ± 12 (4)	98 ± 5 (4)	111 ± 7 (4)
	Dichlorvos	101 ± 15 (7)	114 ± 7 (7)	127 ± 14 (7)
	Ethyl-trichlorfon	81 ± 10 (3)	105 ± 8 (3)	117 ± 18 (3)
Choline acetyltransferase	Saline	1.7 ± 0.5 (6)	21.5 ± 1.6 (5)	27.5 ± 2.1 (3)
	Trichlorfon	2.9 ± 1.1 (4)	30.0 ± 4.3 (4)	21.8 ± 1.6 (4)
	Dichlorvos	1.8 ± 0.6 (7)	27.5 ± 1.3 (5)	24.4 ± 3.5 (7)
	Ethyl-trichlorfon	1.5 ± 0.2 (2)	23.5 ± 2.7 (3)	25.6 ± 4.3 (3)
Acetyl-cholinesterase	Saline	5760 ± 1169 (4)	5691 ± 400 (4)	3283 ± 360 (4)
	Trichlorfon	5023 ± 336 (4)	5515 ± 148 (4)	2568 ± 112 (4)
	Dichlorvos	4662 ± 2793 (7)	6572 ± 848 (7)	3314 ± 585 (5)
	Ethyl-trichlorfon	4759 ± 770 (3)	5275 ± 915 (3)	3385 ± 625 (3)

The dosing regimen given in Table I. Results are expressed as μmol/gm protein × hours and expressed as mean value ± SD (number of offsprings)

iae (30). Dimethoate did, however, not give hypoplasia, but the situation is slightly complicated by the fact that in mammals the amidbond in dimethoate is very rapidly broken by amidases (31). Dimethoate have been reported to give teratogenic effects in rat at the maternal dose of 24 mg/kg (32), but not to mice at 80 mg/kg (33). The teratogenic effects were limited to extra ribs, fused sternebrae and dilated urinary bladder, effects which often occur at low teratogenic doses.

Another possible mediator of the toxic effect of trichlorfon is its main metabolite, trichloroethanol (25). Its precursor chloralhydrate is rapidly converted to trichloroethanol, and both induced high frequencies of aneuploidity in *Aspergillus nidulans* (34). Chloralhydrate has also been shown to induce nondisjunction in spermatogenesis in mouse (35) and to disrupt mitosis in PtK cells in culture by an increase in intracellular calcium (36). Dichloroacetaldehyde is a metabolite of dichlorvos (25) and is also shown to be mutagenic (37). Dichlorvos has been found to be more potent in mutagenic studies in vivo than in vitro and this has been explained by the combined effect from the methoxy group in dichlorvos and an effect of its metabolite dichloroacetaldehyde on DNA. A hypothesis might be that trichlorfon and dichlorvos are teratogenic because of a combination of methylating and clastogenic properties of mother compounds and metabolites. The period between day 40–50 in gestation of guinea pig correspond to the period with brain growth spurt (38). This would be expected to be the most sensitive period for an agent acting on DNA. Effects outside this period may be hard to detect. Also in the period after 50 days of gestation, DNA repair enzymes may play an important role.

It is as yet not decided whether trichlorfon or dichlorvos may cause neurotoxic effects in humans. Trichlorfon is widely used in human medicine to treat infection with *Schistosomiasis haematobium*. The dose applied is 5–15 mg/kg 3 times with 2 weeks interval. There have been no reports of teratogenic effects. There are several objections to the Hungarian report of a teratogenic role of trichlorfon in humans after eating contaminated fish (10). Firstly, the compound used in the fish pool, "Flibol", only had a concentration of 40% trichlorfon. The effects could be caused by other constituents in the compound. Secondly, the effects observed (Downs syndrome) were very different from the effects seen in guinea pigs. The content of trichlorfon per kg in the fish (100 mg/kg) may well have been overestimated. In trials using much higher concentrations of trichlorfon, the maximal concentration in the fish only reached 2.5 mg/kg (I Nafstad, pers. comm.).

In conclusion trichlorfon and its metabolite dichlorvos, both important pesticides, produce brain hypoplasia in two species pig and guinea pig. The effect is not caused by inhibition of acetyl cholinesterase nor by inhibition of brain neuropathy target esterase. The effect may be caused by alkylation of DNA in neurons during early development, possibly at a time when the DNA-repair enzymes are less active in these cells. It is at present not known whether these compounds could cause teratogenic effect in humans. This seems less likely to occur during the treatment of *Schistosomiasis haematobium* nor from the consumption of fish from aquaculture, where only low concentrations may be encountered. It may not be excluded, however, that during exposure to high concentration of these agents (e.g. in a working situation), they constitute a risk for teratogenic effects in pregnant women.

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