

## Cholinergic Pesticides

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### 1. Introduction

The use of protection plant products for the control of pests in agriculture is very ancient: thousands of years ago, Greek and Chinese people knew the insecticide properties of sulfur and arsenic compounds, respectively. The roman Plinio suggested the use of organic insecticides, such as the sedum and marrobium extracts for fighting insects, while Virgil suggested treating the seeds with olive oil to avoid fungine infestation.

After that period, up to the XIX century, the agricultural economy allowed the use of natural remedies, without need of chemical products. Actually, the agricultural sites were relatively small, and with differentiated cultures, so that the effects of infestation of the single cultures were not relevant on the general economy. With the XX century, a new way of regarding to agriculture was diffused, thanks to the availability of large agricultural areas to be used for monocultures. This made necessary the prevention and fight against pests. Paul Herman Muller (1899-1965, Nobel prize for medicine in 1948) first understood the properties of DDT to be effective not only against the common housefly, but also against a wide variety of pests, including the louse, Colorado beetle, and mosquito. This compound was extensively used also in agriculture, but recently it was banned in several Countries cause of its long persistence in environment. High persistence pollutants have been called POPs (Persistent organic pollutants). Persistence is a dangerous feature, because it causes accumulation in the environment and possible bioaccumulation in the organisms.

The studies on toxicity of DDT and other organochlorine insecticides (dieldrin and heptachlor) and the ascertainment of their interference in the endocrine system caused their ban in the US in 1972 (Mellanby, 1992) and in Europe (Council Directive 79/117/EEC\* and Regulation EC No 850/2004 of the European Parliament and of the Council).

Banning organochlorine agents caused an increase in the use of organophosphate and carbamate (CB) pesticides. Organophosphate (OP) is the general name for the esters of phosphoric acid. Organophosphates are the most diffused organophosphorus compounds, and are also the basis of a number of pesticides and insecticides worldwide used and

poured into the environment in the amount of hundred tons every season. These compounds are easily synthesized, and their hemi life lasts from some days to some months in the Laboratory, at room temperature. The discovery of their effects on living organisms was made by the German chemist Willy Lange and his graduate student, Gerde von Krueger (cited by Khurana & Prabhakar, 2000), who first described the effects on cholinergic nervous system. This discovery inspired German chemist Gerhard Schrader (Nobel prize in 1948) in the 1930s to experiment with these compounds as insecticides at company IG Farben. Along these studies, he discovered Tabun, an enormously toxic organophosphate compound towards a number of organisms, including man. Thus, the potential use of OPs as chemical warfare agents induced the Nazi government to develop organophosphate nerve agents (Buckley et al., 2004). In that period the G series of weapons, which included Sarin, Tabun and Soman, was produced. These weapons were not used during World War II. British scientists also synthesized diisopropyl fluorofosphate (DFP), during the war. After World War II, American companies gained access to information from Schrader's laboratory, and began synthesizing organophosphate pesticides in large quantities. Parathion was among the first marketed, followed by Malathion and Azinphosmethyl. These compounds and their formulate derivatives are used for a wide range of aims: from chemical weapon, to pest control and also medical compounds (anxiolytic, antispasmoic, regulators of eye pressure, etc.) (\*part of information obtained from Wikipedia)

Carbamate pesticides (Aldicarb, Carbaryl, Carbofuran, and their formulate derivatives are also largely employed for agricultural, garden and even domestic pest control, and are proposed for substitution of pyrethroid and organophosphorus compounds against *Anopheles* in Third Countries (Akogbéto et al., 2010). The first synthesized and used carbamate is Carbaryl, which was commercialized in 1956. Their persistence in the environment is short, but the persistence is higher in aquatic medium. Thus, environmental effects are exerted mainly on fish and aquatic organisms.

In addition, a new generation of neurotoxic compounds, with effect on a part of the cholinergic system, is represented by neo-nicotinoids, of which the most known is Imidachloprid

## 2. Mode of action of cholinergic pesticides

Organophosphorus and carbamate compounds exert their neurotoxic activity by inhibition of cholinesterase activities (acetylcholinesterase, AChE, E.C. 3.1.1.7 and pseudo cholinesterase, BChE: E.C. 3.1.1.8) and, consequently, the status of the cholinergic neurotransmitter system. These enzymes are modulators of the cholinergic signaling, as their function is exerted by removing the signal molecule acetylcholine (ACh) from its receptors (see the review of Hayes, 1991 for organophosphates and of Fischel, 2008, for carbamates). Consequently their inhibition causes an overflow of ACh at receptor sites, that in turn affects intracellular responses driven by both nicotinic and muscarinic receptors. In this way, neurotoxic compounds may cause alteration of all functions of the cholinergic neurotransmission system, and of other neurotransmitters, whose release is regulated by the pre-synaptic ACh receptors. These insecticides are strongly suspected to cause damage to the human health, and clearly they do in case of acute intoxication, when people gets in contact with high doses, generally for accidents, or occupational causes. But up to date a few data are present about the possibility of subtle chronic (low-dose, long-term) damage due to aerosol diffusion, or to residuals in crops and vegetables, possibly reinforced by the co-

formulated compounds and /or traces of other pollutants, such as other neurotoxic substances, heavy metals, hydrocarbons, or else. On the other hand, the no-effect concentration for man (NOEC), indicated by the pharmaceutical firms and databases, is not surely ascertained, because it is obtained by experimental exposure of animals, generally rats or mice, and then by estimating it as several fold lower. Moreover, very few is known about the possible bioaccumulation in the body, which is different between models and also is subject to individual variability. In addition, the doses that do not affect adults may strike heavily embryonic differentiation, which represents a very sensitive stage of the organism life. The signs and symptoms of carbamate poisonings are similar to those caused by the organophosphate pesticides. The carbamate's principal route of entry is either by inhalation or ingestion or secondarily by the dermal route. Dermal exposure tends to be the less toxic route than inhalation or ingestion. For example, carbofuran has a rat oral LD<sub>50</sub> of 8 mg/kg, compared to a rat dermal LD<sub>50</sub> of greater than 3,000 mg/kg, making it much more toxic when ingested. The carbamates are hydrolyzed enzymatically by the liver; degradation products are excreted by the kidneys and the liver. Respiratory depression combined with pulmonary edema is the usual cause of death from poisoning by carbamate compounds. As with organophosphates, the signs and symptoms are based on excessive cholinergic stimulation. Unlike organophosphate poisoning, carbamate poisonings tend to be of shorter duration because the inhibition of nervous tissue acetylcholinesterase is reversible, and carbamates are more rapidly metabolized (Fischel, 2008).

**The pharmacology of OPs and carbamates** has been extensively studied, and the differences are resumed as follows: 1) OPs irreversibly link the AChE molecule by the phosphate group (Guo et al., 2003) thus preventing the ingress of ACh in the active site of the gorge, while carbamates compete for the substrate acetylcholine (ACh), allowing reversibility of the effects (Minneau, 1991); 2) OPs can leave residuals in the environment, while carbamates only leave small inorganic molecules (Fishe, 2008).

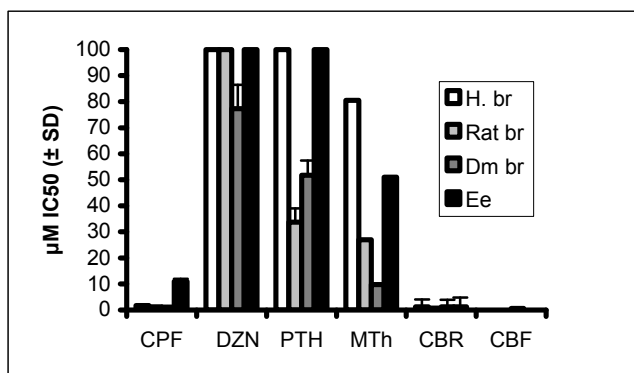


Fig. 1. (Made by Rakonczay, in the frame of the EC project SENS-PESTI, QLK4, and reported in the paper by Aluigi et al., 2005). Effect of organophosphates and carbamates on acetylcholinesterase activity from different sources. IC<sub>50</sub> values are reported as means  $\pm$  SD, of 3-6 independent experiments with triplicate samples. Preincubation with inhibitor was 30 min, the inhibitors were solved in MeOH, final concentration of the MeOH in the incubation mixture was 0,5%. Purified electric eel AChE was purchased from Sigma, by use of three different lot numbers of enzyme preparations.

3) Most of OPs are soluble in lipids, and this allows passage through the cell membranes and accumulation in fat tissues.

### Metabolites toxicity

The metabolites of both carbamates and Ops are more than tenfold active in ChEs inhibition than their parent molecules (Sultatos, 1994, Aluigi et al., 2005) Actually, the link between the oxon derivatives and the serine residuals present in the gorge of AChE molecule is much more persistent, and it is said that oxonized OP compounds definitely "kill" the AChE molecule (Sultatos, 1994).

The IC<sub>50</sub> for CPF was between 1 and 10  $\mu$ M, depending on the different organisms sensitivity; the IC<sub>50</sub> of DZN and PTH was between 30 and more than 100  $\mu$ M (over the diagram scale). The carbamates showed IC<sub>50</sub> around 1  $\mu$ M for all the organisms, including the purified Electric eel AChE, used as a control.

Student's t-test with 2-tailed significance values showed:

Drug	H Br vs	P value	H Br vs	P value	H Br vs	P value
CPF	rat br	< 0,002:	<i>Dm br</i>	< 0,05	Ee	< 0,0001
DZN	rat br	NS	<i>Dm br</i>	< 0,01	Ee	NS
PTH	rat br	< 0,0001	<i>Dm br</i>	< 0,0002	Ee	NS
MTh	rat br	< 0,0001	<i>Dm br</i>	< 0,0001	Ee	< 0,0004
CBR	rat br	< 0,0004	<i>Dm br</i>	NS	Ee	< 0,04
CBF	rat br	< 0,0008	<i>Dm br</i>	< 0,0004	Ee	< 0,0001

Table 1. Significance of the different effect of the drugs on human AChE molecules vs neural tissue of different organisms. CBF = carbofuran; CBR= carbaryl; CPF = Chlorpyrifos; CBR; DZN=diazinon; MTh = malathion; PTH = fenthion; H br = human brain; *Dm br*= *Drosophila melanogaster* brain; rat br = rat brain; Ee= electric eel purified cholinesterase (Sigma)

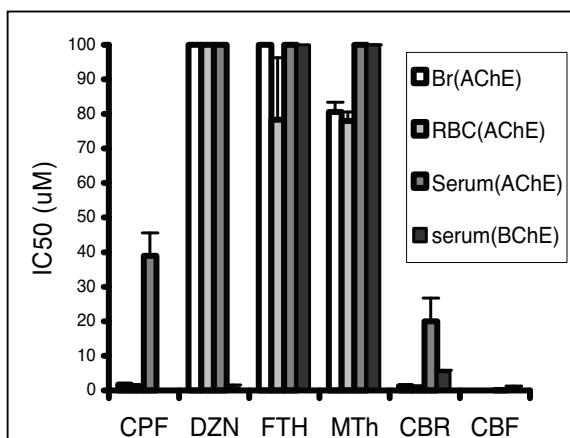


Fig. 2. IC<sub>50</sub> of OP and CB compounds on AChE and BChE of different human tissues

### 2.1 Primary and secondary targets of toxicity

AChE activity is the primary, but not the only one target of cholinergic compounds toxicity: actually, according to Casida & Quistad (2004), secondary non-AChE targets are represented by inhibition of pseudocholinesterases, and ACh receptors, such as the muscarinic ones, that can be affected directly, besides the effect mediated by AChE inhibition. This causes sometimes contradictory effects, such as increase of AChE activity in the affected organs (Aluigi et al, 2005; Aluigi et al., 2010a, 2010b) showing a sort of paradox effect.

The effect of some cholinergic inhibitors is different not only among different organisms, but also among different brain parts. Actually, Rakonczay and Papp (2001) also found that after an acute (4 h) treatment with an irreversible cholinesterase inhibitor organophosphate, metrifonate (100 mg:kg i.p.), the activities of both acetyl- and butyrylcholinesterase were inhibited (66.0–70.7% of the control level) in the rat brain cortex and hippocampus. There were no significant changes in the acetyl- and butyryl-cholinesterase activities in the olfactory bulb, or in the choline acetyltransferase activity in all three brain areas.

**The third class of cholinergic substances** (neonicotinoids) are insecticides which act on the neuromuscular system of insects with lower toxicity to mammals. Neonicotinoids are among the most widely used insecticides worldwide, because they affect a molecular form of nicotinic ACh receptor, which is typical of insects. The mode of action of neonicotinoids is similar to the natural insecticide nicotine, that (like ACh) activates the response of nicotinic ACh receptors, but is not cleaved by ChEs. In insects, neonicotinoids cause paralysis which leads to death, often within a few hours. The main concern for the use of these insecticides is due to a possible connection to honey bee Colony Collapse Disorders and generally for the disappearance of pollinator insects. According to what is reported in the literature, no damage may be exerted on man, cause of the specific binding to insect receptors, but recent data may suggest a certain caution. Preliminary experiments show competition between Imidachloprid and  $\alpha$ -BuTx, a snake venom from *Bungarus multicinctus*, selectively binding to the  $\alpha$ -7 subunit of the mammalian nicotinic receptor.

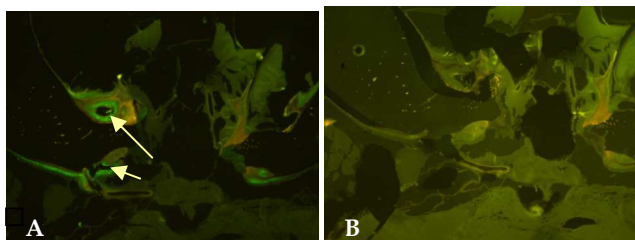


Fig. 3. Cross section of bee heads, embedded in Kulzer 7100 resin, sectioned 3  $\mu$ m thick. Both the sections were incubated 1 h in the dark with  $10^{-8}$ M FITCH-conjugated  $\alpha$ -BuTx, in PBS pH 7.4. A: untreated bee; B: bee pre-exposed for 10 min to  $10^{-5}$ M Imidachloprid. (Thesis of Dr. Guglielmo Castagnoli, 2009).

### 2.2 Persistence in the environment and crops

The persistence of these compounds in the environment is generally considered fairly short, but evidences have been found that in sediments they may remain for long times, as occurred in the river Rhine (Dauberschmidt et al, 1996) where lethality of fish, mollusks, and aquatic birds lasted for months and kilometers downstream. According to Ragnarsdottir

(2000) an OP pesticide presenting short half-life in the laboratory increases to one year in conditions of low pH and temperature. The same author reported that OPs are detected in soils years after application, probably due to sorption of the OPs to soil particles, making them unavailable for microbial metabolism (Ragnarsdottir, 2000). As far as living organisms are concerned, the effects of such compounds may last much more, because the AChE of blood may be affected up to several months (see the case report Romero et al. 1989).

### **2.3 Studies in USA, South America, Australia, Eastern Countries**

These two classes of pesticides are directed towards both insects and other small pest organisms, and act similarly. They interfere with cholinergic transmission in the nervous system of their target, and affect human health because AChE is a common enzyme, active in the nervous system of all the living organisms, and involved in cell-to-cell communications, including those leading embryonic development and differentiation. For this reason, their effects on human health have been studied more intensively in countries such as USA, South America and Australia, and even eastern Countries, such as India, where the agricultural sites cover big areas, and agriculturists represent an important part of the population. In Europe only a few researcher groups work on this argument.

Some commonly used organophosphates in these countries include malathion, methyl parathion, chlorpyrifos, azinphosmethyl, and diazinon. Common N-methyl carbamates include aldicarb and carbaryl (*List of chemicals evaluated for carcinogenic potential*. U.S. EPA Office of Pesticide programs, 26 August 1999).

Anyway, in these countries also, the study of the effects of low-dose exposure to contaminants is rather neglected, because OPs and CBs, introduced to replace organochlorines, are generally shorter-lived in the environment, and more acutely toxic. This way of regarding the problem causes an underestimate of the possible contact with consumers, due to residuals in the crops, and also to the fact that after collection, during transport, the merchandises are packed and again treated with pesticides (e.g. bananas from Costa Rica are packed in plastics with chlorpyrifos and shipped to European markets: personal communication from distributors).

### **2.4 Europe**

The main bulk of studies in Europe are represented by environmental diagnostics: i.e. identification of the presence of contaminants in the environment by use of AChE biochemical detection as a bio marker. Two of these projects were recently supported by the European Community (Project Reference: ACHEB QLK3-2000-00650; SENS\_PESTI, QLK4-CT2002-02264). The last one involved our group, and most of the reported results were obtained along development of this project (2003-2006), and after, as a proceeding of work. Here we report some of the results obtained in the frame of SENS-PESTI, together with other outstanding reports available in the literature. The studies worldwide are an enormous number, cause of the socio-economical relevance of the topic, thus our report cannot be as complete as I would like.

## **3. Human diseases possibly related to occupational exposure**

### **3.1 Acute intoxication**

This term refers to the immediate sensible effects (generally within 24 hours) of a particular dose of cholinergic pesticide on human health.

The exposure to such high doses of the contaminants is generally caused by accidents such as the one occurred in the river Rhine in November, 1986 (Dauberschmidt et al., 1996), which caused an ecological disaster, including lethality of fish, mollusks, and aquatic birds for months and kilometers downstream. The effects of acute intoxication are mainly exerted on the nervous system, through the hyper activation of receptors, causing peripheral nervous symptoms, also called *cholinergic crisis*, up to death (Jamal, 1997). The symptoms are due to muscarinic receptors (cardiac arrhythmia, salivation, lacrimation, hypotension, respiratory problems, headache, dizziness), and to nicotinic receptors, causing paralysis, muscular cramps, and titanic contraction of muscles (Aardema et al., 2008). This crisis is sometimes followed by a more dangerous late onset of symptoms, such as asystole, which may appear after weeks, when the patient is released from the Hospital (Chacko and Elangovan, 2010). The effects of the acute intoxication are well known and classified as well as the first aid practice and antidotes, such as oxime and atropine (see Sultatos, 1994, Aardema et al., 2008, for extensive reviews).

### 3.2 Chronic intoxication from low continuous or repeated doses

At long term, nervous system disorders may occur: for instance, it was discussed for many years and now definitely established, from epidemiological studies in California, that in areas where pesticides are spread, the incidence of certain neurodegenerative diseases is increased (Davis et al., 1978; Betarbet et al., 2000). Respiratory effects may lead to aggravation of pre-existing conditions such as asthma (Underner et al., 1987). Actually, it is known that one of the effects of OPs is exerted on broncho constriction (Reeves et al., 1999). Carbamates such as Carbaryl, may also cause morphologically deformed sperms etc. Between 1991 and 1996, California EPA reported 3, 991 cases of occupational poisoning by agricultural pesticides (O'Malley, 1997). Domestic use of pesticides may cause symptoms that are similar or identical to those caused by other illnesses, so that chronic pesticide poisoning is often misdiagnosed.

In particular, neurotoxic pesticides effects are directed towards embryonic development as shown by experiments on invertebrates and vertebrates differentiation (Sherman, 1966; Morale et al., 1998, Pesando et al., 2002, Aluigi et al., 2005, 2010a). Numerous case reports and case series present various combined severe congenital anomalies following occupational or accidental exposure of pregnant women to OP pesticides (Romero et al., 1989, Soreq and Zakut, 1990).

Chronic intoxication is due to prolonged or repeated exposure to low doses of pesticides. This is slow and may cause subtle health effects, and every body may be exposed, for the diffusion of aerosols, or by consuming agricultural products (in some agricultural sites, a survey of 1997 revealed that the large-leaf vegetables on the market were found to contain from 0.3 to 0.007 mg/Kg organophosphate residues (Ligurian EPA, personal communication).

Chronic health effects from pesticides are problematic to study in humans, because most people are exposed to low doses of pesticide mixtures, symptoms appear late in time, and delayed health effects are difficult to link to past exposures.

#### 3.2.1 Cancer facts

Among the effects on human health, several are known or suspected: cancer facts, such as inheritable gene amplification suspected to cause tumors in families of agriculturiers (Soreq

and Zakut, 1990; Shapira et al., 2000); multiple recent reports link hairy cell leukemia (HCL) with pesticide exposure, in particular with organophosphate exposure (Clavel et al. 1996). More recent studies (Cabello et al, 2001) have demonstrated a relationship between malathion and parathion and the induction of mammary tumors (possibly related to the function of these two compounds as endocrine disrupters, as all the liposoluble organic compounds are potentially able to interfere in steroid hormones reception). In human adults, Gorell et al. (1998) reported about the possibility that neurotoxic pesticides may induce neurodegenerative diseases in the population of agricultural areas.

In addition, the increased permanence of ACh at the receptors, caused by the impairing of AChE by ChE inhibitors, may act as a coadjuvant of tumor progression. Actually, in some tumour types, following activation of nicotinic and/or muscarinic receptors (Dodds et al., 2001; Minna, 2003); the MAP Kinase cascade is activated, driving cell proliferation (Ukegawa et al., 2003; Trombino et al., 2004). MAPK are important signal molecules, leading to cell growth and proliferation (Davis et al., 2009). At the same way, in the lung cancers following hyperactivation of nicotinic receptors, cell death regulation is compromised, thus causing the enhancement of cell proliferation. This can explain why tumour progression is enhanced by tobacco smoking (Cooke & Bitterman, 2004).

The researchers group lead by H. Soreq recently provided epidemiological and molecular evidence that the "readthrough" AChE, (AChE-R), a variant form of AChE induced by stress, and in particular by stress induced by pesticides, can cause inheritable diseases, including some cancers, in agriculturators' families (Soreq & Zakut, 1990; Shapira et al., 2000). During the last years this Researchers group provided evidence of the involvement of such a stressed form of AChE in anxiety (Ofek et al., 2007; Adamec et al., 2008), inflammation (Dori et al., 2007) and also in the modulation of beta-amyloids (Berson et al., 2008; Buznikov et al., 2008).

### 3.2.2 Neurological facts

Due to the fact that AChE is directly involved in the modulation of signals during the primary neural induction from the notochord to the neurogenic ectoderm (Aluigi et al., 2005) toxicological implication of AChE inhibitors on this process appears evident (Brimijoin and Koenigsberger, 1999). All the anticholinesterase drugs, by increasing the cholinergic tone of receptors, can cause neuropsychological defects (Colosio et al., 2009); organophosphates cause impairment of neural development (Aluigi et al., 2005), as well as of memory and psychomotor speed, and affective symptoms such as anxiety, irritability and depression (Frost, 2000), visual-spatial deficits, and from recent experiments OPs are suspected to be involved in new variant transmissible spongiform encephalopathy (Purdey, 1998).

Neurological development in children is particularly at risk of disruption. Animal studies demonstrate periods of vulnerability, particularly to anticholinesterase, during early life (Karczmar et al., 1970). Recent evidence that AChE may play a direct role in neuronal differentiation supports these findings (Biagioni et al., 2001).

### 3.2.3 Reproductive and developmental anomalies

Reproductive (Nelson, 1990) and developmental facts (Chanda and Pope, 1996; Aluigi et al. 2005, 2008, 2010a, 2010b) were also demonstrated to be caused by maternal, embryonic or differentiating cells exposure to neurotoxic pesticides.

Moreover, in animal experiments, AChE activity was shown to be involved in limb bud chondrogenesis (Falugi & Raineri, 1985), and the amount of AChE and ChE present in blood



was shown to be depleted for several months in persons exposed to organophosphate pesticides in USA (Romero et al., 1989). In one case, the consequence of the depletion of AChE activity (due to professional OP exposure) in the maternal blood, lasting throughout the pregnancy, caused the birth of a baby with only one eye, brain and heart anomalies, who died after some days (Romero et al., 1989).

During the last decades, neurotransmitter systems (Buznikov, 1990; Buznikov and Shmukler, 1996) and in particular the cholinergic systems (Drews, 1975; Minganti et al., 1981; Fluck et al., 1980 and Falugi, 1993) have been found responsible for cell interactions leading early development. In particular, molecules belonging to the cholinergic system, whose presence and amount is regulated by AChE and BChE activity, were found to exert a neurotrophic effect (Filogamo & Marchisio, 1971), and a strong input to neurogenesis and axon differentiation (Biagioni et al., 2001). In this light, a danger to early neural development of human fetus is strongly suspected as well as to the later establishment of neural function in children and in adults.

From recent studies OPs are suspected to be involved in adolescent behavioural disturbance (Bouchard et al., 2010) attention-deficit/hyperactivity disorder (ADHD) in children 8 to 15 years of age. Cross-sectional data from the National Health and Nutrition Examination Survey (2000-2004) were available for 1139 children, who were representative of the general US population. From a structured interview with parents, one hundred nineteen children met the diagnostic criteria for ADHD. These children also presented high levels of DMAP, a metabolite of thionophosphates, supporting the hypothesis of a relationship between exposure to OP drugs and ADHD.

### 3.2.4 Developmental anomalies

The developmental anomalies occurring after exposure to cholinergic drugs generally regard tissues and organs where in normal conditions AChE activity is mainly localized. At early stages, which Buznikov called "pre-nervous" and Drews called "embryonic", the effects are linked to the role of AChE and the molecules to it related, in cell-to-cell communication, generally due to intercellular messages, mediated by ionic fluxes and intracellular ionic changes. AChE activity has been found in vertebrate embryos (e.g. chick embryos, Aluigi et al., 2005) since the first stages, localized in the Hensen's node, and successively in the wall of the primitive streak, in the somites in the notochord, and in the floorplate of the neural tube, i.e. in temporal windows where cell-to cell communication awaking gene expression and consequent cell movements (Drews, 1975) take place. Thus, the presence of AChE activity is related to 3 classes of developmental events: I: during gamete maturation, activation and interaction (Angelini et al., 2004; Angelini et al., 2005); II: during the early development of invertebrate and vertebrate embryos. In this case cholinergic molecules are located mainly in moving cells and tissues engaged in relevant morphogenetic events, such as gastrulation and limb bud differentiation, and are often co distributed with special extracellular matrix molecules such as fibronectin (Aluigi et al., 2005) and laminin (Johnson et al., 2003); III: during inductive communications between mesenchyme and other tissues such as the limb bud development (Falugi and Raineri, 1985). The cholinergic system thus seems to be a multifunctional cell communication system. It appeared early during evolution as a regulator of intercellular communications mediated by ion dynamics (In *Paramecium primaurelia* it is related to the mating behaviour of single eukaryotic cells: Delmonte Corrado et al., 1999), before becoming involved in highly specialized communication structures, such as synapses and nerve endings.

## Vertebrate models

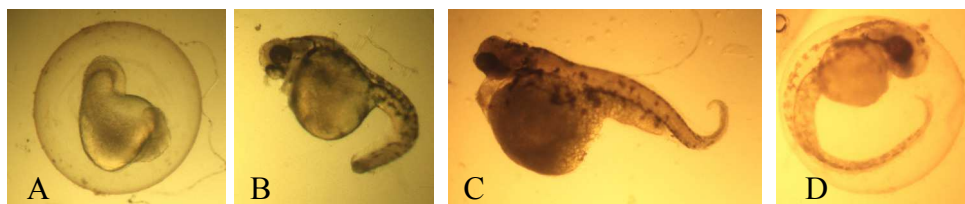


Fig. 4. Developmental anomalies in zebrafish embryos exposed at the mid-gastrula stage to different concentrations of fenthion: A:  $10^{-5}$ M; B and C:  $10^{-6}$ M; D: unexposed sample.

The curled trunk and tail are common features for a number of neurotoxic pesticides: the same aspects were found by interlaboratory calibration of the test by the team of Prof. Layer after exposure to chlorpyrifos and carbamates (in the frame of SENS-PESTI) (unpublished data).



Fig. 5. A: 24 h incubated chick embryo. The Hensen's node (H) and the ridges of the primitive streak appear positive for the AChE reaction; B: 36h incubated chick embryo: the head neural fold (arrow), the first neural tube and the primitive streak are positive to the AChE reaction, revealed by dark reaction products. C: 48h control embryo; D: chick embryo, exposed to  $10 \mu\text{M}$  DZN at 24 h incubation (corresponding to the A image) and sampled at 48h incubation, showing anomalies in the proximal part of the body (head did not develop nor differentiated and heart is double, because mesoderm movement was impaired, so that the two simple tubes forming heart failed to join anteriorly) This kind of anomalies was found to be caused by all the cholinergic pesticides, with high sensitivity as compared to the sensitivity of adults (see Aluigi et. Al., 2005).

## 4. Mechanisms of action

### 4.1 Apoptosis

#### 4.1.1 Alternative models: cultured cells.

In terms of gene expression analysis, cDNA microarray studies showed that the most statistically significant pathways affected were related to cellular death and cell proliferation. (Catalano, 2007). Actually, we (Aluigi et al, 2010b) had evidence that the OP

compounds may affect differentiation and cell proliferation/death of NTERA2-D1 cells (NT2). The NT2 cell line, which was derived from a human teratocarcinoma, exhibits properties that are characteristics of a committed neuronal precursor at an early stage of differentiation. Its property to express a whole set of molecules related to the cholinergic neurotransmission system, including active acetylcholinesterase (AChE, EC 3.1.1.7) makes it a good alternative model for testing the effects of neurotoxic compounds, such as organophosphorus (OP) insecticides, whose primary target is the inhibition of AChE activity.

Non-neuromuscular AChE expression was also found in a number of cell lines upon induction of apoptosis by various stimuli (Zhang et al., 2002). The induction of AChE expression was determined by cytochemical staining, immunological analysis, affinity chromatography purification, and molecular cloning. The authors found the AChE protein in the cytoplasm at the initiation of apoptosis and then in the nucleus or apoptotic bodies upon commitment to cell death. Sequence analysis revealed that AChE expressed in apoptotic cells is identical to the synapse type AChE.

Pharmacological inhibitors of AChE prevented apoptosis. Furthermore, blocking the expression of AChE with antisense inhibited apoptosis.

As the mechanisms of the relation between AChE and apoptosis are still rather obscure, we carried on bioassays, by blocking the AChE activity in cultured human cells, NTERA2-D1.

NT2 cells exposed to the OP insecticide diazinon at concentrations ranging between  $10^{-4}$  and  $10^{-5}$ M showed a time-dependent enhancement of cell death. When exposed at  $10^{-6}$ M diazinon showed higher cell viability than control samples up to 72 h, followed by a decreasing phase. The cell death caused by the exposures showed a number of features characteristic of apoptosis, including membrane and mitochondrial potential changes. We suggest the hypothesis that such behaviour is due to a dynamic balance between activated and blocked acetylcholine receptors that in turn trigger electrical events and caspase cascade. (Fig.6)

## 4.2 Calcium dynamics

### 4.2.1 Models for developmental effects: Invertebrates (sea urchin)

For this research, we mainly used, besides cultured cells, sea urchin early development as a model. Sea urchin is one of the few organismic models approved and validated by the European Agency for Alternative models. Actually, sea urchin embryonic development has been studied for over a century, and the complex nets of intercellular communications leading to the different events are well known, as well the possibility for environmental molecules and their residuals to interfere with such communications, causing developmental anomalies. In particular, the main goal of toxicologists since several years has been to establish a correlation between the cell-to-cell communications occurring during different developmental events and the signals occurring during neurogenesis, with the aim to pursue a mechanistic understanding of these processes and their deviations caused by stressors from different sources. By use of this model, at different developmental stages, we established that neurotoxic insecticides may affect calcium dynamics since fertilization events (Pesando et al., 2002). The biological effects of *Basudin* (an organophosphate compound containing 20% *Diazinon*), *Diazinon* (Dzn, a thionophosphate), *Carbaryl* and *Pirimicarb* (carbamates) on the early phases of sea urchin development were thus investigated. Morphological, biochemical, histochemical and immuno histochemical analyses were performed both during embryo and larval development.

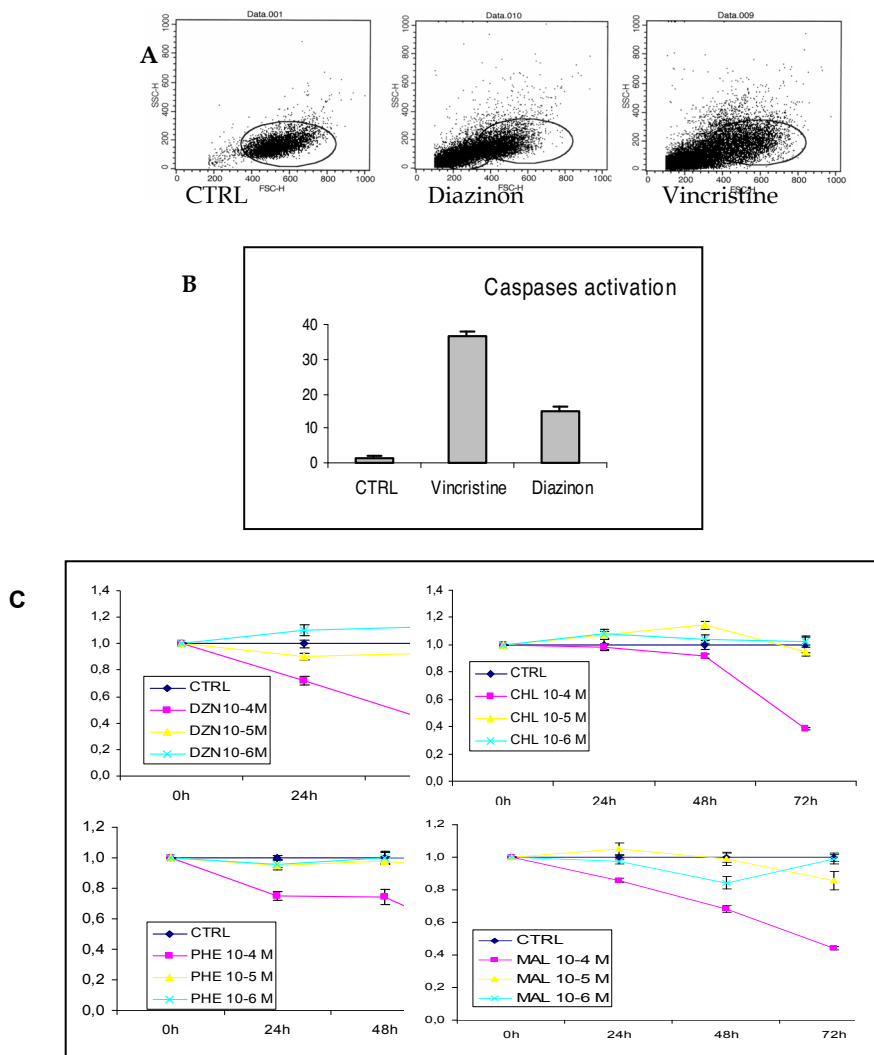


Fig. 6. (from Aluigi et al., 2010b). A: cytofluorimetry showing apoptosis of NT2 cells, controls and exposed to 10  $\mu$ M DZN and vincristine, as a positive control. The amount of apoptotic cells was  $C < DZN < vincristine$ . (B) The same trend was seen in caspase expression. C shows the percentage of survival (Y axis) along time (X axis, each unit corresponds to 24 h), at concentrations ranging between 100 and 1  $\mu$ M. (cell viability was measured by use of the MTT method)

#### 4.2.2 Invertebrate models: the sea urchin, *Paracentrotus lividus*

For the morphological effects on fertilisation and first cleavages, the effective concentration of insecticides was found to be 10<sup>-4</sup>M, while for further stages concentrations between 10<sup>-5</sup> and 10<sup>-7</sup>M were effective. 10<sup>-3</sup>M of any of these insecticides totally arrested development.

This results depend on the fact that no cholinergic molecules are involved in fertilisation, as we demonstrated successively (Harrison et al., 2002). Thus, the high dose (that is about IC50, according to the previously shown data of Rakonczay) may cause a general toxicity effect, not related to cholinergic molecules. On the other hand, Casida and Quistad (2004) reported a number of non-cholinergic secondary targets, and this could explain the general toxicity. In contrast, effects revealed at the molecular level, such as lectin binding and AChE activity seem much more sensitive, and may reveal anomalies at the chronic exposure concentrations ( $10^{-7}$ M). At these low concentration, an effect was seen at later stages, during the larval growth, on cell proliferation and larval plasticity (Aluigi et al., 2010a), as larvae exposed to CPF and PTH low levels along the whole development showed longer perioral arms and fastened metamorphosis. Concentrations as high as  $10^{-5}$  and  $10^{-6}$ M blocked larval development and, when used to expose larvae next to metamorphosis, caused immature forms of juveniles, lacking skeletal structures. The effects of AChE inhibition on the skeleton formation were also seen in the early larvae (Ohta et al., 2009). As other Authors (Hoogduijn et al., 2006) found an effect on human osteogenic stem cells, we can speculate some involvement in human osteoporosis for direct toxicity on AChE that has also been reported to be present in pre-cartilage nodules of chick embryos (Falugi and Raineri, 1985). In the case of sea urchin, arm elongation, sustained by calcium carbonate skeletal rods, may be due to different causes: the first may be due to slowing of the ciliary movement, and consequent starvation of the larvae. According to Fenaux et al. (1988), perioral arms elongate for increasing the ciliated area that brings food to the mouth. The second explanation is that enhancement of arm growth could be due to a direct effect on muscarinic receptors, which are distributed along the arms at the basis of the cilia.

Exposure to diazinon in a particular developmental window (10 min after fertilization) also caused the formation of exogastrulae (Fig.7).

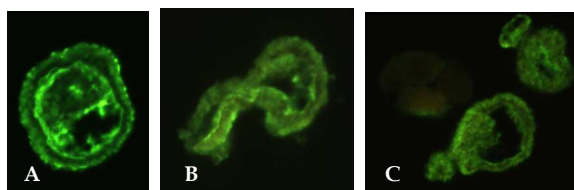


Fig. 7. A: control gastrula; B, C, different aspects of exogastrulae exposed to  $10^{-5}$ M diazinon. The green immunofluorescence shows the localization of muscarinic receptors (primary antibody obtained from Chemunex, Fr) Unpublished images.

The final target of OP poisoning, as we have seen above for other models, is the regulation/disregulation of particular genes: Also this was studied by using sea urchin as a model. In this model, we recorded the effects of OP exposure (in particular diazinon) on the localization of a regulatory protein that is immunologically related to the human OTX2. The severe anomalies and developmental delay observed after treatment at  $10^{-5}$  M concentration are indicators of systemic toxicity, while the results after exposure to the inhibitor at  $10^{-6}$  M concentration suggest a specific action of the neurotoxic compound. In this case, exposure to diazinon caused partial delivery of the protein into the nuclei, a defective translocation that particularly affected the blastula and gastrula stages. Therefore, the possibility that neurotoxic agents such as organophosphates may disregulate expression of outstanding proteins is taken into account.

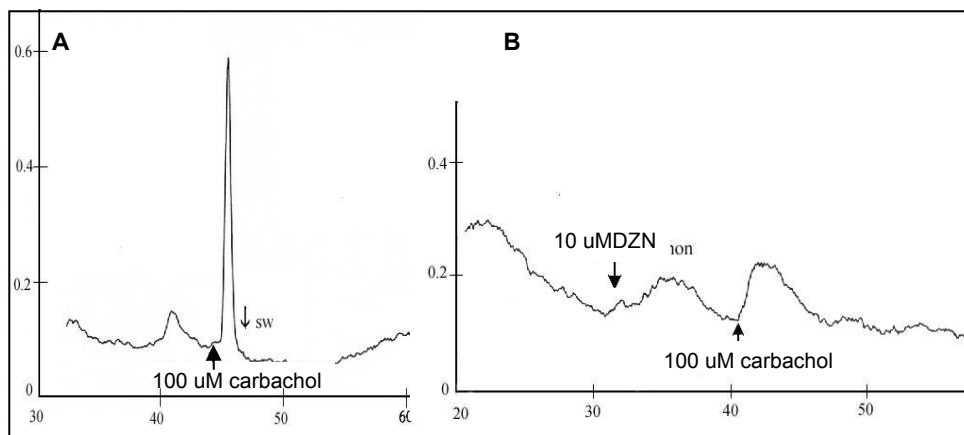


Fig. 8. A: *Litechinus pictus* zygotes were exposed to 100  $\mu$ M carbamylcholine (carbachol), a cholinomimetic agonist of the muscarinic receptors. Exposure to carbachol is followed by a spike of fura2-dextran fluorescence. B: exposure to carbachol is preceded by exposure to 100  $\mu$ M diazinon, no spike follows the exposure to 100  $\mu$ M carbachol. Y axis represents fluorescence (scale units = 0,2) units; X axis represents time (scale units 30 sec). These experiments were performed by Dr Harrison P. in the laboratory of Prof. Whitaker, MJ, and published in the paper Harrison et al., 2002.

Actually, from the zygote stage, stimulation of ACh receptors may evoke calcium spikes, anticipating those related to the nuclear breakdown (Harrison et al., 2002). In this event, muscarinic drugs were proved to have a prominent role. As a consequence of intracellular  $[Ca^{2+}]$  alteration, all the calcium related intracellular dynamics are altered, including delivery of transcription factors to the nuclei.

By use of sea urchin early developmental stages, and DZN exposure at different concentrations, evidence was provided that cytoplasmic dynamics were perturbed and in particular the delivery of the OTX2 protein, which in mammals plays a role in forebrain development. We (Aluigi et al., 2008) submitted the hypothesis that this effect could be due to altered calcium dynamics, which in turn alter cytoskeleton dynamics: the asters, in fact, appear strongly positive to the OTX2 immunoreaction. (Aluigi et al., 2008). In this work, sea urchin early developmental stages were used as a model to test the effects of the organophosphate pesticide (diazinon) on the regulation of gene expression by immunohistochemical localization of the regulatory protein against the human OTX2. Egg exposure to diazinon did not affect fertilization; however, at concentrations  $10^{-5}$ - $10^{-6}$  M, it did cause developmental anomalies, among which was the dose-dependent alteration of the cytoskeleton. Coimmunoprecipitation experiments showed the link between cytoskeletal tubulins and the OTX2 protein, thus justifying the partial delivery to nuclei.

In addition, Pesando et al. (2003) showed that, during embryonic development, the treatment with organophosphates slowed the rate of early mitotic cycles down, affected nuclear and cytoskeletal status as well as DNA synthesis. From the gastrulation stage onwards, the main effects were exerted on the rate of primary mesenchyme cells migration, larval size, perioral arm length, and acetylcholinesterase activity distribution, thus deregulating the cholinergic system, which modulates cell-to-cell communication mediated by the signal molecule acetylcholine.

### 4.3 Biosensors

We found that the effects of cholinergic insecticides exposure were more drastic in developing organisms than in adult tissues. Although the experiments on developing embryos were used at concentrations of the drugs including those indicated in the labels as under threshold (NOEL), effects were found on developmental anomalies. This is because development is a multi-phase event, where each stage depends on the previous ones, thus amplifying also the small defects that in adults are easily corrected or healed.

In order to protect not only human health, but also environment and next generations, at present, a great deal of effort is concentrated on creating "biosensors", capable of perceiving neurotoxic compounds in the environment, as well as in food and water. Most of the biosensors are represented by devices that have the capacity to measure, with high sensitivity, the activity of acetylcholinesterase in the presence of suspected inhibitors and in particular OP or carbamate compounds.

These high-technology instruments can measure the presence and amount of neurotoxic compounds in environmental matrices, or in rough material and elaborated foods. The biosensors used for this purpose are generally based on highly sensitive molecular forms of AChE, immobilised in devices capable of recording changes in activity in real time, and by transferring them to screens or other recording devices (Crew et al. 2004), or by use of mutated bacteria or yeast (Wu et al. 2002).

All those biosensors are very good tools to evaluate the degree of exposure of people, by analysing blood, urine, or else. Along development of the SENS-PESTI project, in the Laboratory of by Hagen Thielecke (the Fraunhofer Institute for Biomedical Engineering (IBMT), based on micro technology), a new kind of biosensor is was studied, which is able to evaluate the effects of exposures on living organisms, and their health risks, by evaluating living cells and tissues responses to exposure. In this case, it is possible to evaluate not only the effects on the primary target AChE, but also any response evoked by secondary targets of pesticides (Abdallah et al. 1992; Sultatos 1994). Such a biosensor has the capacity to translate the effect of neurotoxic pesticides in living cells into electrical signals by using microtechnological devices for measuring e.g. the alteration of ion fluxes or their intracellular concentration. The advantages of this biosensor are represented by the fact that complex cell response is taken into account, and that the AChE molecules and the ACh receptors are in their natural environment, and follow their natural transduction cascades up to the cell response.

The employment of such devices is at present innovative, as well as complying with the International bioethical concerns. Actually, it may solve some controversial points, such as:

1. The problem of experiments on animals, which are more expensive, besides causing pain, which is particularly evident in higher organisms.
2. The improved knowledge of developmental biology, within the emerging knowledge that neurotransmitter molecules are not limited to neuromuscular structures, but are generally involved in cell-to-cell communication, leading to interaction between developing cells and tissues.
3. Exporting the results between different organisms, including man, by comparing the effects of exposures on animal tissues (zebrafish, sea urchin, xenopus early embryos, that are considered bioethical by ECVAM, ICCVAM and other International Institutions (dealing with toxicity test validation) with the effects on human cultured cells and adult stem cells.

4. Allowing us to establish conversion parameters among the different cell sources, in order to use the most suitable and available for each situation of risk assessment.

## 5. Present problems and possible solutions

The main problem for the use of pesticides is the confusion at present existing in this field. Very huge numbers of researches are carried out all over the world, but the results are often contradictory, and scarce information is given to the final users, i.e. the agriculturists and consumers. The number of accidents occurring every year is high, although a legislation exists about the use of safety items and safety provisions. Moreover, the Thematic Strategy on the sustainable use of pesticides adopted in 2006 by the European Commission aims at filling the current legislative gap regarding the use-phase of pesticides at EU level through setting minimum rules for the use of pesticides in the Community, so as to reduce risks to human health and the environment from the use of pesticides. For the moment, the Commission has proposed to restrict the scope of the Framework Directive to plant protection products. Directive 2009/127/EC amending Directive 2006/42/EC with regard to machinery for pesticide application: Machinery used for applying pesticides in European farms, orchards, vineyards, parks and gardens will be more environmentally friendly, thanks to an amendment to the Machinery Directive published in November 2009.

Everyone who uses pesticides, has the responsibility to ensure their correct and effective use. To help them, the EC provides guidance on best practice in the use of pesticides in a number of ways. Nevertheless, up to date, the label is the main source of information on the safe and effective use of a product. The product label must always be supplied with the container. Additional information may also sometimes be supplied as a separate leaflet within the boxes containing the products.

Actually, besides intentional self-poisoning or terrorist attacks, the more usual way to be intoxicated by neurotoxic pesticides is the practice of agriculturists, mainly in the moments when they dissolve high amounts of powders or use sprays without safety aids.

Epidemiological studies suggest that chronic exposure may increase susceptibility to neurodegeneration diseases (Betarbet et al., 2000), not only for agriculturists but also for housekeepers who take care of their clothes and safety aids. Thus all the family of agriculturists is involved in learning how to prevent exposure. Bystanders and consumers are also a target of chronic toxicity (Keifer & Mahurin, 1997), and no information is provided on the markets about the date of last application of plant protection products on vegetables. So, a new trend is emerging in consumers about the use of organic food. For pregnant women and children, the benefits are worth the higher price (Jurosek et al., 1999)

For this reason it is needed a careful information and use of safety aids in the correct way. When used responsibly, pesticide products provide many benefits such as promoting affordable and abundant food supplies. To ensure the safety of the environment and human health, pesticides are also heavily regulated. The Environmental Protection Agency (EPA) is the government body responsible for regulating pesticides and assessing risks associated with these chemicals. This includes evaluating whether pesticides pose an unreasonable risk to humans and the environment and requiring pesticide registrations when applicable

It is essential that all the information is read carefully and understood before a pesticide is used because it informs the user of the safe and proper use of the product. To this aim, it is requested a great effort in the future for training of agriculturists, to provide them with a clear picture of risks and ways to avoid them.



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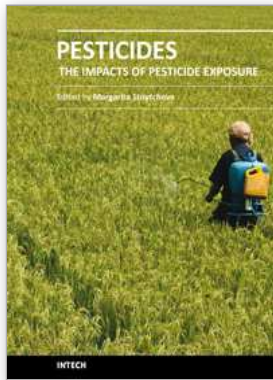
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## **Pesticides - The Impacts of Pesticides Exposure**

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Pesticides are supposed to complete their intended function without “any unreasonable risk to man or the environment”. Pesticides approval and registration are performed “taking into account the economic, social and environmental costs and benefits of the use of any pesticide”. The present book documents the various adverse impacts of pesticides usage: pollution, dietary intake and health effects such as birth defects, neurological disorders, cancer and hormone disruption. Risk assessment methods and the involvement of molecular modeling to the knowledge of pesticides are highlighted, too. The volume summarizes the expertise of leading specialists from all over the world.

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