Opinion on possible links between BSE and Organophosphates used as pesticides against ecto- and endoparasites in cattle - Report and opinion adopted at the Scientific Steering Committee meeting of 25-26 June 1998

Remark: the present document contains opinions adopted by the Scientific Steering Committee of the European Commission, which is a neutral and independent scientific body.

Readers should keep in mind that the present report and opinion only address the scientific aspects of the risk assessment of the issue (e.g., identification of hazards, levels of infectivity in the starting materials and final products, etc.). The risk management and policing aspects related to the implementation of an opinion, are not dealt with.

Executive Summary

In its opinion on Organophosphates and TSE's of 16 May 1997, the Multidisciplinary Scientific Committee (MDSC) concluded that no relation existed between the use of and exposure to organophosphates and the occurrence of BSE.

In January 1998, the Scientific Steering Committee (SSC) was requested to evaluate and possibly amend the MDSC's opinion of 16 May 1997 in the light of possible additional evidence and scientific literature that meanwhile may have become available. More precisely, it was invited to provide its opinion on the hypothesis that there is a link between the use of some organophosphates, especially Phosmet, and the initiation of BSE by the formation of delayed neuro-excitatoric proteins as a consequence of the phosphorylation of the PrP in the foetuses of the treated cows to the toxic \( PrP^{Sc} \) protein.

The Committee carefully analysed the available evidence. It confirmed the opinion of the MDSC that there is no evidence for such link to exist.

Opinion

I. Framework and mandate

In its opinion on Organophosphates and TSE's of 16 May 1997, the Multidisciplinary Scientific Committee (MDSC) concluded that no relation existed between the use of and exposure to organophosphates and the occurrence of BSE.

The hypothesis of a link between the use of and exposure to organophosphates and the occurrence of BSE was forwarded in two papers by Purdey (1996) (22; 23) on "The UK-epidemic of BSE: Slow Virus or Chronic-Pesticide-Initiated Modification of the Prion-Protein? Part 1: Mechanism for a Chemically-Induced Pathogenesis / Transmissibility. Part 2: An Epidemiological Perspective".

In January 1998, the Scientific Steering Committee (SSC) was requested to evaluate and possibly amend the MDSC's opinion of 16 May 1997 in the light of possible additional evidence and scientific literature that meanwhile may have become available. More precisely, it was invited to provide its opinion on the hypothesis that there is a link between the use of some organophosphates, especially Phosmet, and the initiation of BSE by the formation of delayed neuro-excitatoric proteins as a consequence of the phosphorylation of the \( PrP \) in the foetuses of the treated cows to the toxic \( PrP^{Sc} \) protein.

The key primary question upon interpretation of the mandate is whether there are indications that organophosphates (e.g. Phosmet) bind to brain prions similarly as they do to the acetylcholinesterase (AchE), following by aging which results in a denaturation of the proteins. These denaturated phosphorylated prions would then possibly and hypothetically be precursors of BSE.

II. Scientific background information on organo-phosphorous compounds
II.1. Uses of Organophosphates and Sources of Consumer Exposure

Commercial compounds usually summarized under organophosphates comprise esters, amides or thiol derivatives of phosphoric, phosphonic, thiophosphoric and thiophosphonic acids. About one hundred active ingredients are or have been used in several hundred products against arthropode pests. In addition, highly toxic substances out of the group have been developed as chemical warfare agents (Tabun, Soman, Sarin, VX). (1).

The major use of organophosphates is as agricultural insecticides covering the whole range of crop growing and storage. Most commonly known ingredients are e.g. Azinphos-methyl, DDVP, Dimethoate, Fenitrothion, Malathion, Metasystox, Parathion. The insects to be controlled include the whole range of arthropode pests. Organophosphates and carbamates represent the second generation of agricultural insecticides after DDT and Drin-insecticides. Due to the high mammalian toxicity of the organophosphates and resistance development in certain pests to be controlled, they are to some extent followed by the third generation, the pyrethroids.

A further important area of use is in animal husbandry against arthropode exo-and endoparasites. Here only active ingredients at the lower end of the mammalian toxicity can be used (e.g. Bromphenphos, Dichlorvos (DDVP), Chlorvinphos, Fenthion, Phosmet (Fosdan, Imidian, Prolate, is one of the several organophosphates used on cattle against grub, horn fly or others. Their use on cattle is world-wide.

Other minor uses of organophosphates, e.g. additives, are of no relevance.

Most organophosphates are of low persistence. Nevertheless, there are residues on crops or stored food as well as in meat and in meat products. These residues require that acceptable daily intakes for the active ingredient and maximum residue limits for food commodities have been set assuring consumer safety. Although lipophylic (water solubilities typically in the range of 25-100 mg/l) organophosphates are not considered to bioaccumulate due to fast range metabolism/low persistence. The bioaccumulation factor of Phosmet e.g. in fish is about 50.

Only triesters of phosphoric acid are considered in the present report.

II.2. Toxicodynamics and Toxicokinetics

Both the toxicodynamics (mechanism of action) and toxicokinetics (distribution, metabolism, etc.) of OP's are largely explained by their biochemical characteristic of interacting with esterases and proteases (2). Esterases have been ranked into two main categories: those inhibited by Op's, B-esterases, representing potential targets for toxicity, and A-esterases which hydrolyze OP's, thereby being involved in detoxification. OP's interact with either esterase as substrates: B-esterases after the formation of a Michaelis complex are phosphorylated and the reactivation is either very slow or it does not occur at all. A-esterases, on the contrary, hydrolyze OP's and their catalytic center is rapidly restored.

Moreover, a further reaction might occur on phosphorylated B-esterases, a phenomenon called aging, involving the loss of a group attached to phosphorus and leading to the formation of a negatively charged irreversibly phosphorylated enzyme. On a given enzyme, rates of reactions depend on the chemistry as well as on chirality of the inhibitor.

Any given B-esterase is inhibited by various OP's at different rates. Also rates of reactivation and aging of phosphorylated enzymes are variable, depending on the phosphoryl residue which occupies the catalytic center. Therefore, the degree of inhibition of an esterase and its duration at the site depend both on the enzyme itself and on the chemistry of the OP. For instance, while OP's inhibit acetylcholinesterase (AChE) at variable concentrations, both spontaneous reactivation and aging depend on the phosphoryl residue bound to the active site. As a result of AChE phosphorylation by different OP's, this residue can be the same.

II.3. Toxic Mechanisms

II.3.a. Cholinergic Overstimulation

The molecular mechanism of cholinergic toxicity involves the interaction of OP's with AChE (3), an elongated
molecular structure formed by heterologous subunits, is localized in the outer basal lamina of the synapse. A single gene encodes the catalytic subunits of AChE and the three-dimensional structure of AChE has been determined (4). Both substrate and inhibitor react covalently with the enzyme in essentially the same manner because acetylation of the resin residue in the active center of AChE is analogous to phosphorylation. However, in contrast with the acetylated enzyme, which rapidly gives acetic acid and restores the catalytic center, the phosphorylated enzyme is stable.

Calculated turnover rates, i.e. the number of molecules hydrolyzed per minute by one molecule are as follows: 300,000 for acetylcholine and 0.008 for OP's. Spontaneous reactivation of enzyme may require several hours (dimethoxy) or does not occur at all (secondary, such as DFP, or tertiary alkyl groups). The loss of one alkyl group, occurring through the non-enzymatic process of aging, further enhances the stability of the phosphorylated enzyme.

AChE's crystal structure reveals that the anionic moiety of AChE's, thought to attract the quaterny nitrogen of the substrate, is misnamed because it contains at most one negative charge. It has been proposed instead that the quaternary moiety of acetylcholine binds chiefly through interactions with the aromatic residues which line the walls and floor of the gorge.

When blocked by the phosphoryl residue, the serine group of the catalytic center is no longer able to participate in the hydrolysis of acetylcholine. Thus the neurotransmitter accumulates, its action is enhanced and given the widespread distribution of cholinergic neurotransmission, toxic effects of OP's will involve parasympathetic, sympathetic and somatic motor component of the PNS and also the CNS (5). Signs and symptoms include lacrimation, hypersalivation, bronchial hypersecretion and bronchoconstriction, urination and defecation, skeletal muscle fasciculation and twitching, ataxia, respiratory failure, convulsions, hypothermia and death. Death is due to respiratory failure resulting from the combination of these effects.

The interaction of acetylcholine with either muscarinic or nicotinic receptors leads to various biochemical effects on second messenger systems (6) and eventually to the toxic response. Single doses of OP's do not affect brain muscarinic receptors (7) whereas repeated exposures may reduce both their density and affinity for specific ligands (8). Reduction in muscarinic receptors shows regional specificity (9), reflecting either differences in duration or intensity of cholinergic stimulation, or a selective access of the inhibitor. Reductions of high-affinity brain nicotine-binding sites have also been found after chronic cholinergic stimulation (10). Symptoms of excessive cholinergic stimulation are gradually reduced during chronic OP exposure, despite significant inhibition of AChE, the development of this tolerance has been in part associated with down-regulation of muscarinic receptors caused by prolonged AChE inhibition and acetylcholine stimulation (11).

II.3.b. Delayed Polyneuropathy

Single doses of certain OP's cause a central-peripheral distal sensory-motor axonopathy known as organophosphate-induced delayed polyneuropathy (OPIDP) (12,13,14,15,16).

The molecular target is thought to be a protein in the nervous system called Neuropathy Target Esterase (NTE). High inhibition of NTE (> 70%) in the nervous system, measured within hours after dosing, correlates with the delayed onset of clinical signs of OPIDP 10-20 days later. OPIDP is caused by certain, but not all OP's, providing they inhibit NTE above the threshold. Doses causing OPIDP depend on the OP, the route of administration, the species and other factors.

However from the practical point of view, it is important how the dose causing OPIDP compares with that causing cholinergic toxicity (17). This concept, represented numerically by the ratio LD50/neurotoxic dose, allows comparisons of the potential of OP's to cause OPIDP. Thus a ratio LD50/neurotoxic > 1 discriminates OP's causing OPIDP at doses which do not cause cholinergic toxicity from those which cause OPIDP only if animals are treated against cholinergic symptoms (ratio < 1). All commercial OP insecticides have a ration of < 1 and most have a ratio < 0.1. Therefore among NTE inhibitors, cholinergic toxicity is the limiting factor for OPIDP development.

Histopathology of OPIDP has been described for several species (18, 19). The morphological hallmark is axonal degeneration of motor and sensory fibers characterized by focal nerve varicosities and paranodal remyelination located in the distal but not terminal axons. There is no evidence of death of corresponding neurons, but varying degrees of chromatolysis occur in proportion to the severity of neuropathy. Ultrastructural studies show aggregation and accumulation of neurofilaments and neurotubules as well as proliferation of smooth endoplasmic reticulum, particularly
in proximity to nodes of Ranvier. Lesions are distributed both in the CNS (spinal cord) and PNS (peripheral nerves of legs mostly. The degree of pyramidal involvement predicts the pathogenesis of OPIDP. If only peripheral nerves are involved the neuropathy is reversible within several months whereas if CNS is involved, spasticity is permanent.

II.4. Phosmet

II.4.a. Identity and Use

Phosmet is a non-systemic OP insecticide used on both animals and plants. It can be used in the treatment of warble-fly in cattle and also as an active ingredient in some dog collars.

II.4.b. Chemical Structure

The structure of Phosmet is given in the following Figure. Its IUPAC name is O-O-dimethyl-S-phthalimidomethyl-phosphorodithioate.

Figure

II.4.c. Metabolism and Fate (20):

In humans and animals:

- Phosmet shows a very rapid kinetism with a very quick absorption, distribution and elimination. Radiolabeled Phosmet was excreted predominantly in the urine: by the time of sacrifice (120 hours after treatment) 79% had been excreted in the urine and 19% in the faeces, while very little was expired as 14CO². Tissue levels of radiolabel were low, especially in fat and the gonads. In an other labeled study in rats very little label (1-2%) was detected in the carcass 96 hours after treatment. The lowest concentrations of label were found in bone and fat and the highest in the skin. There were no data mentioned for brain levels.

- It crosses the placenta - a single dose of 70mg/kg in goats showed no more residues in the milk after 24 and 48 hours.

- Cattle fed silage with an average residue level of 19 ppm for 2 months showed no residue levels in the milk above the detection limit of 0.01 ppm - dietary levels of 20-100 ppm showed no residues in the tissues higher than 5 ppb.

- Metabolic breakdown in very quick. A proposed metabolic pathway is given in the following figure. Phtalamic acid and phtalic acid and their esters are the most important metabolites. Phtalamic acid would have been deaminated to phtalamic anhydride and hydrolysed to phtalic acid.

(Figure)

Environmental Fate:

- Phosmet is rapidly broken down in soil. The compound persists longer in dry soil than in moist soil. Breakdown is also faster under basic conditions.

- In water it is rapidly broken down by hydrolysis and by sunlight (photolysis). Under alkaline conditions (pH 9) the half-life is as short as 16 hours - in a neutral solution (pH 7) the half-life is 18 hours and under acidic conditions (pH 5) the half-life is within 9 days.

- Plants break down phosmet quickly, primarily by oxidation and hydrolysis. Washing and blanching can reduce residue levels by 50 to 80%.

II.4.d. Effects on Enzymes (20)

Erythrocyte and brain cholinesterases are more sensitive to Phosmet in rats than is plasma cholinesterase. Rat aliesterases are more sensitive to inhibition by Phosmet than is acetylcholinesterase.
A dose of 10 mg/kg orally administered has no effect on plasma or erythrocyte cholinesterase activities at either time or on brain cholinesterase activity at 24h. However 4 hours after treatment with this dose, brain cholinesterase activity was inhibited by 14% in males and 21% in females.

II.4.e. Toxicological Effects (20)

- Acute Toxicity:
  Moderately toxic by ingestion - moderately to highly toxic through the skin - very high toxic through inhalation.

Oral LD50 in rats is 113 - 369 mg/kg bw - in mice 23-50 mg/kg bw.

The compound appears to be more toxic to many domestic animals (cattle, sheep and goats): LD50 from 25-50 mg/kg bw., than for experimental animals.

- Chronic Toxicity:
  In rats: NOAEL of 1-2 mg/kg/day
  In dogs: NOAEL of 1 mg/kg/day

Cattle: 1-2 mg/kg for 8 weeks provoked a decrease of blood enzyme activity.

In rabbits: during 3 weeks applied to their skins showed high mortality at doses of 300-600 mg/kg/day.

- Delayed Neurotoxicity was not observed in chickens, the most sensitive animal species known.

- Reproductive Effects:
  In rats: 2.0 mg/kg bw for the first generation -4.0 mg/kg for the second and third generations did not provoke negative reproductive effects.

In rabbits: 10-60 mg/kg dermally and orally 3 weeks before mating and on 18 consecutive days of gestation showed no effects on reproduction parameters.

Conclusion: Phosmet did not shown negative effects on reproduction.

- Teratogenic Effects:
  In rabbits: 10-60 mg/kg for 3 weeks gave no birth defects. A NOAEL was estimated to be 35 mg/kg

In monkeys: 8-12/kg for days 22-32 of gestation gave no birth defects.

In rats: 30 mg/kg between day 9 and 13 of gestation produced an increase in brain damage (hydrocephaly) in 33 out the 55 embryos examined. With lower doses (1-5 mg/kg) such effect was not seen.

Conclusion: No convincing evidence for teratogenic effects has been found in rabbits or monkeys, whereas in rats only, at high doses of 30 mg, some effect was seen, probably due to maternal toxicity.

- Mutagenic Effects

In bacteria: no any mutation test was positive except in two tests with one strain of Salmonella Typhimurium (reverse mutation).

In animal cells: two mouse lymphoma tests were performed with a positive result, one (forward mutation at the tk locus)
in the absence of metabolic activation and one (sister chromatid) in the absence of metabolic activation (< 0.1 mg/ml) and one in the presence of metabolic activation (0.008-0.040 mg/ml).

Conclusion: its mutagenic potential is rather unclear but certainly not proven.

- Carcinogenic Effects:

In rats: 1-20 mg/kg/day for 2 years showed no differences but there were too few rats at the end of the test. In another study no tumours were seen that were attributable to treatment with Phosmet.

In mice: no treatment-related changes in organ-weights or macroscopic or microscopic appearance were seen, except in the liver. There was an increase in the incidence of liver adenomas (25/50) in males at a dose of 100 ppm. Liver adenomas were found in 13/60 controls, 10/60 at 5 ppm, 14/60 at 25 ppm and 27/60 at 100 ppm. The prevalence of liver adenomas in the group given the highest dose was reported to be comparable to that in historical controls. No increase in the incidence of liver tumours was seen in females.

III. Comments on the papers of Purdey (1996)

III.1. Paper 1. The mechanism for the pathogenesis / transmissibility

The author proposes a hypothesis in which it is said that exposure of the bovine embryo to specific high-dose lipophylic formulations of organophosphate insecticides was the primary trigger that initiated the UK's Bovine Spongiform Encephalopathy epidemic. The mechanism should be a covalent binding with phosphorylating and aging serine, tyrosine or histidine active sites on fetal CNS prion protein. Once this abnormal prion protein isoform agent is initiated, any stress event ensuing in adult life induces a nerve-growth-factor-mediated synthesis of normal cellular prion protein isoform that aggregates to abnormally phosphorylated isoform. The abnormally phosphorylated isoform PrP Sc is left corrupted by an extra charged phosphate group. In so far the summary of the hypothesis.

Comments from the SSC:

Purdey (1996a, 1996b) combines a series of hypotheses with proven and unproven biochemical and biophysical features. Some criticisms are formulated hereafter:

- Phosmet is very quickly metabolized and eliminated with no significant tissue accumulation except in fish. Moreover it has been proven that Phosmet residues have no delayed neurotoxicity in chickens, the most sensitive animal species. Such a proof is not demonstrated in the paper.

- No mention is made of any reference or study which proves the presence of a receptor site on the surface (membrane) of the prion protein. It is not enough to speculate on the presence of a serine molecule in order to propose also a covalent binding of OP's on a prion protein. The whole hypothesis is speculative.

- According to the paper, the hypothesis for the trigger effect on brain prion proteins by Phosmet is supported by its structure-relationship with Thalidomide, a very well known teratogen.

- Thalidomide:

The chemical structure of Thalidomide is shown in the following figure.

(Figure)

This product is very intensively hydrolysed in vivo to form secondary, tertiary and quaternary hydrolysis products. These hydrolysis products however do not appear to possess significant teratogenic activity.

There is also a very different sensitivity to Thalidomide between the different animal species. So the teratogenic effects of Thalidomide are not still proven in cattle.
An intact Phtalimide or Phtalimidine group appears to be essential for teratogenic activity. Thalidomide teratogenicity was dominantly restricted to skeletal malformations mostly of the limbs.

- Phosmet

It seems indeed that Phosmet contains a phtalimide moiety. Hence the conclusion that Phosmet may have identical toxicological properties as Thalidomide and that this gives an explanation for the so-said CNS protein toxicity.

However extensive structure-activity studies have been carried out with over 60 compounds stereochemically related to Thalidomide (under which Phosmet (21). Only Thalidomide itself and three other analogs (See figure) are clearly teratogenic in rabbit, the most sensitive experimental animal.

(Figure)

- Finally before the whole proposed hypothesis can scientifically be accepted as real and the link between the use of OP's and BSE can scientifically be accepted in the pathogenesis of TSE's, the possible affinity and convavalent binding of OP's for PrP protein should be shown. Therefore real studies are lacking in his paper.

Conclusion: DFP (di-iso-propyl fluorophosphate) is an OP which causes both cholinergic toxicity and delayed polyneuropathy, approximately at the same dose. It has been used as a model OP to ascertain if these chemicals, including phosmet, might bind to prion proteins. Since no binding was detected it is unlikely that OP's would be capable of modifying this protein, either directly or with a mechanism other than phosphorylation.

III.2. Paper 2: An epidemiological perspective

This paper tries to elucidate the flaws in the hypothesis that BSE originated from alterations in the way that scrapie-contaminated cattlefeeds were manufactured in the UK. The whole epidemiological evolution of BSE is explained in the frame of the hypothesis already described in the first paper.

His conclusion is that both timing, distribution and dynamics of usage of these specific OP's (a.o. Phosmet) correlates with the epidemiology of BSE.

Comments from the SSC:

The first paper provides a scenario in which the use of Phosmet as treatment for warbles and the epidemiology of BSE are put in relation with each other. But data and numbers are lacking and the scenario is rather speculative and anecdotal. The exact number of farms where Phosmet was indeed applied in cattle and the exact number of really diagnosed BSE cases in the same farms are not provided.

Conclusion: The SSC concludes that the paper is no a true epidemiological study as it is not scientifically founded.

IV. Opinion

On the basis of the elements and evaluations presented in the above report, the Scientific Steering Committee confirms the opinion of 16 May 1997 of the Multidisciplinary Scientific Committee and concludes that there is no scientific evidence of possible links between BSE and organophosphates used as pesticides against ecto- and endoparasites in cattle.

V. Acknowledgements

The present report and opinion adopted by the Scientific Steering Committee is substantially based on the work of a working group chaired by Dr.E.Vanopdenbosch. Special thanks are addressed to Professor .Dr.M.Debackere for his major role in the preparation of the present document. The other members of the working group were Professor Dr. M.Lotti, Professor Dr. W.Klein and Professor Dr.Ed.Med.F.Kemper.
VI. Literature


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25. **Kemper, F., 1998.** Letter of 8 January 1998 To Dr.E.Vanopdenbosch, rapporteur of the working group on Possible links between organophosphates and BSE.


27. **Lotti, M.,1997.** Letter to the secretariat of the Multi-Disciplinary Scientific Committee on the UK epidemic of BSE.

28. **Poulsen, E., 1997.** Comments on the postulated relation between effects of organophosphorous compounds and the disease in cattle, BSE. for the attention of the Multi-Disciplinary Scientific Committee.