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In Vivo Investigation of Dietary Exposure to 5 Pesticides

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Preface

The studies presented in this report were performed at the Danish Veterinary and Food Administration, Institute of Food Safety and Nutrition. The studies were financed by The Danish Environmental Protection Agency. In the period from 2000-2003 two *in vivo* dietary exposure studies were performed as repeated dose 28 days oral toxicity studies in rats. A dose-response study with the pesticide chlorpyrifos and a combined exposure study with the pesticides alphacypermethrin, bromopropylate, carbendazim, chlorpyrifos and mancozeb were performed.

Animal studies, clinical observations, food and- and water consumption, behavioural- and functional tests were performed by Grethe Østergaard and Otto Meyer at Department of Toxicology, Section for Biology. Euthenization, macroscopic- and microscopic pathology was performed by Ole Ladefoged and Helene Jacobsen at Department of Toxicology, Section for Pathology. Henrik Frandsen and Henrik Rye Lam at Department of Biochemical and Molecular Toxicology performed analysis of plasma- and brain acetylcholinesterase activity. Mette Erecius Poulsen at Department of Chemical Contaminants measured pesticide content in diets and plasma.

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Sammendrag og konklusion

Vi udsættes alle dagligt for små mængder pesticider gennem den mad vi spiser. Pesticidrester blev fundet i ca. 30% af dansk produceret frugt og i ca. 70% af udenlandsk produceret frugt i de stikprøver, som Fødevaredirektoratet undersøgte i 2001. I de udenlandske produkter findes desuden små rester af pesticider, der er forbudte at anvende i Danmark.

Den mulige sundhedsskadelige effekt af pesticider er traditionelt blevet vurderet på basis af det enkelte pesticids egenskaber. Der er kun få dyreforsøg, der belyser risikoen ved indtagelse af en kombination af pesticider og andre kemiske stoffer, og dermed kun få data til rådighed for risikovurdering. De forsøg, der findes, viser, at kombinationseffekter af to eller flere kemiske stoffer ved lave koncentrationer enten ikke kan måles eller højest er additive. Der er endnu ikke en international accepteret metode til sundhedsmæssig risikovurdering af blandinger af kemiske stoffer. Fødevaredirektoratet anvender en metode, hvor mængden af alle pesticidrester som er fundet i en fødevare lægges sammen. Den samlede indtagelse af resterne vurderes udfra det mest giftige pesticid, nemlig det der har den laveste "Acceptable Daily Intake" (ADI). I de tilfælde, hvor ADI for det mest giftige pesticid overskrides, beregner man indtaget af hvert enkelt af de påviste pesticider ved et såkaldt Hazard-Index. Hazard Index er et mål for det samlede indtag af pesticider fra en prøve. Indtaget af hvert stof vægtes i forhold til stoffets ADI. Hazard Index beregnes som summen af forholdet mellem indtaget af hvert stof og dets ADI .

Formålet med forsøgene i denne rapport var at bidrage med data om mulig kombinationseffekter ved indtagelse af flere pesticider der findes som rester i fødevarer.

Fem pesticider (alphacypermethrin, bromopropylat, carbendazim, chlorpyrifos og mancozeb) blev valgt. Alle 5 pesticider findes ofte som rester i fødevare-stikprøver undersøgt af Fødevaredirekroratet i Danmark. Rotterne blev i 28 dage fodret med foder, der var tilsat de 5 pesticider i forskellige koncentrationer. I det indledende forsøg blev rotterne fodret med stigende doser af alle 5 pesticider. Dernæst blev der på baggrund af resultaterne lavet et dosis-responsforsøg på et af pesticiderne (chlorpyrifos), og til sidst endnu et kombinationsforsøg, hvor rotterne fik stigende doser chlorpyrifos og en konstant dosis af en kombination af de øvrige 4 pesticider (alphacypermethrin, bromopropylat, carbendazim og mancozeb)

Rotterne i det højest doserede hold i det indledende forsøg blev forgiftet, og viste tegn på påvirkning af i hjernen i form af bevægelsesforstyrrelser og ændret adfærd og dette hold måtte udgå. I det mellemste og lavest doserede hold sås hhv. 12 og 8 % nedsat aktivitet af enzymet acetylcholinesterase i hjernen hos hunner, samt et fald i antallet af hvide blodlegemer hos hanner. Derudover havde det mellemste hold forstørret lever og rotterne virkede mere lydfølsomme og aktive ved aflivningen. Resultaterne var overraskende, så der var behov for en opfølgning af undersøgelsen.

To af pesticiderne, chlorpyrifos og alphacypermethrin, har kendt effekt på hjernen. Chlorpyrifos ved hæmning af enzymet acetylcholinesterase. Alphacypermethrin ved at ændre permeabiliteten af natrium i nervemembranerne og dermed øge stimulering med hjernesignalstoffer generelt. Da chlorpyrifos var det eneste pesticid i blandingen, der påvirker enzymet acetylcholinesterase, og da der var konstateret en hæmning af enzymet i hjernen hos hunner i både det lavest og det mellemste doserede hold, blev det besluttet at fokuserer på virkningen af chlorpyrifos og en mulig kombinationseffekt. Det blev ved et dosis-respons forsøg med chlorpyrifos konstateret, at der ikke var tale om en utilsigtet overdosering med chlorpyrifos i det indlendende forsøg. Der sås ingen effekt på dyrene eller på hjernens aktivitet af enzymet acetylcholinesterase. Endnu et kombinationsforsøg blev udført, hvor chlorpyrifos blev givet i varierende doser mens dosis-niveauet af de fire andre pesticider var konstant. Der sås ingen påvirkning af hjernen eller af hjernens indhold af enzymet acetylcholinesterase i dette forsøg. Fundene fra det indledende forsøg kunne således ikke eftervises. Der var dog andre effekter i rotterne bl.a. vægtændring i leveren, brislen og skjoldbruskkirtlen, ændringer i blodbilledet og ændret histologi i leveren og skjoldbruskkirtlen.

Konklusionen af de 3 dyreforsøg var, at effekter ses hos rotter ved lavere doser, når rotterne udsættes for en kombination af 5 pesticider, end når rotterne udsættes for pesticiderne enkeltvis. En sådan kombinationseffekt viser at en sundhedsmæssig risikovurdering af indtagelse af rester af pesticider er vanskelig.

Den overraskende påvirkning af hjerne- og nervefunktion set i det indledende forsøg kunne ikke genfindes i det opfølgende kombinationsforsøg. Niveauet af mancozeb var kun på 18-26% af det tilsigtede i det opfølgende kombinationsforsøg, hvorfor det ikke kan udelukkes, at effekten på hjernen i det indledende forsøg faktisk var en ægte effekt.

Kombinationseffekter blev set i det opfølgende kombinationsforsøg, i form af øget relativ lever- og skjoldbruskkirtelvægt samt nedsat vægt af brislen i kombinationsgrupperne. Endvidere var der histologisk forandringer i lever og skjoldbruskirtel samt ændringer i både røde- og hvide blodceller i kombinationsgrupperne.

I det indledende forsøg blev det overvejet, om der var en øget omsætning af chlorpyrifos til den aktive metabolit, chlorpyrifos-oxon. Chlorpyrifos-oxon blev målt i det opfølgende kombinationsforsøg. Chlorpyrifos-oxon kunne ikke findes i plasma, hvilket kan skyldes, at der ikke er en øget levermetabolisme af chlorpyrifos. Dog var der desværre problemer med metoden til måling af chlorpyrifos-oxon. Effekterne set i det indledende forsøg kunne også skyldes andre mekanismer, f.eks. ændret toksikokinetik (optagelse, omsætning og udskillelse).

Fra et regulatorisk synspunkt er effekten på hjerne og nerve, set i det indledende forsøg stadig bekymrende, og der er stadig behov for en forklaring. Hvilket af pesticiderne gav anledning til hjernesymptomerne alphacypermethrin, chlorpyrifos eller en kombination?

Der er yderligere behov for at udføre nogle toksikokinetiske studier for de enkelte pesticider og kombinationer af pesticiderne. Toksikokinetiske studier kan belyse mulige vikningsmekanismer, da der kan være ændringer i

optagelse, fordeling, omsætning og udskillelse af et af pesticidene i kombinationen.

Summary and conclusions

We are daily exposed to a number of pesticides through the food we eat. In fruit pesticide residues were found in approximately 30% of Danish produced fruit and in approximately 70% of the fruit produced outside Denmark in the test samples examined by the Danish Veterinary and Food Administration. In fruit and vegetables produced outside Denmark residues of pesticides, that are forbidden to use in Denmark, are also found.

The possible adverse health effects of pesticides have traditionally been evaluated on the basis of the individual properties of single substances. There are few animal studies on combination of pesticides and other compounds, and therefore only few data available for risk assessment. Those available show that the combined effect of two or more chemical substances at low concentration is either not detectable or at most additive. There is still no internationally agreed method for evaluation of combination of compounds. The Danish Veterinary and Food Administration uses as a first cut-of an approach of summing all pesticide residues and compare the intake based on the acceptable daily intake (ADI) for the most toxic pesticide involved for the risk assessment. If the summarized ADI for all the pesticides exceeds the most toxic compounds ADI, a Hazard-Index is calculated. A Hazard-Index is calculated as the sum of the theoretical intake of the individual pesticides in the food item in relation to the ADI of the individual pesticides.

The purpose of the studies in this report was to provide further data on possible combined effects of pesticides that may occur as residues in food.

Five pesticides (alphacypermethrin, bromopropylate, carbendazim , chlorpyrifos and mancozeb) were chosen. All five pesticides are often found as pesticide residues in test samples of food examined by the Danish Veterinary and Food Administration. The rats were fed the pesticides in various concentrations through the diet for 28 days. In the initial study the 5 pesticides were all given in increasing doses. Due to the results of the initial study a dose-response study of chlorpyrifos was done and at last a combined exposure study was performed where the rats were fed increasing doses of chlorpyrifos and a constant dose of the other four pesticides (alphacypermethrin, bromopropylate, carbendzim and mancozeb).

The rats in the high dose group of the initial study were intoxicated with signs of disturbances of the central nervous system (CNS) with incoordinated movements and changed behaviour, this group was terminated. In the mid and low dose groups an inhibition of 12 and 8% in brain acetylcholinesterase in female rats was found and a decrease in the number of white blood cells in male rats was found. The mid dose group had increased liver weight and appeared more sensitive to sounds and were more active at autopsy. These result were surprising and there was a need for further investigation of the results.

Two of the pesticides, alphacypermethrin and chlorpyrifos, have known effects on CNS. Chlorpyrifos by inhibiting the enzyme acetylcholinesterase. Alphacypermethrin by changing permeability of sodium in nerve membranes

causing general stimulation of neurotransmitters. Since an inhibition of brain acetylcholinesterase was found in female rats in the intial study in the low and mid dose group, we focused on chlorpyrifos and a possible combination effect. A dose-response study of chlorpyrifos was performed. There was no effect in the animals and no inhibition of acetylcholinesterase in the brain. Another combination study was performed where chlorpyrifos was given in increasing doses and the four other pesticides kept at the same level. No effect was found on CNS or on brain acetylcholinesterase. The results from the initial study could thus not be confirmed. However, other effects were seen with weight changes of liver, thymus and thyroid gland, changes in haematology and in histology of the liver and thyroid gland.

The conclusion of the 3 animal studies was that effects are seen at lower levels when rats are exposed to the five pesticides in a combination than when the rats are exposed to the individual pesticides. Such a combined effect shows that risk assessment of health effects of residues of pesticides is complicated.

The unexpected effect on CNS and on inhibition of brain acetylcholinesterase seen in the initial study could not be repeated in the second combination study. However, the level of mancozeb in the diet in the last combination study was only 18-26% of the intended, which means that the effect on CNS in the initial study still might be a true effect.

Combination effects were seen in the last combination study, as there was weight increase in the relative liver weight, the thyroid gland weight and a decrease in the weight of the thymus in the combination groups. There were changes in the histology of the liver and the thyroid gland and changes in both white and red blood cells in the combination groups.

In the initial study a possible increase in liver metabolism of chlorpyrifos to the active metabolite chlorpyrifos-oxon was considered. However, increased liver metabolism of chlorpyrifos to chlorpyrifos-oxon could not be confirmed, since chlorpyrifos-oxon was not found in plasma. However, there were some problems with the method used to measure chlorpyrifos-oxon. The effects seen in the initial study might be through other mechanism e.g. change of toxico-kinetics (absorption, distribution, metabolism and elimination).

From a regulatory point of view the CNS symptoms seen in the intial study still give reason to concern, and there is a need to explain these findings. Which of the pesticides in the combination gave rise to the CNS symptoms - alphacypermethrin, chlorpyrifos or a combination?

There is furthermore a need to perform some toxicokinetic studies of the single compounds and the compounds in combination. Toxicokinetic studies might elucidate possible modes of action, since there might be changes in absorption, distribution, metabolism and elimination of a single compound in the combinations.

1 Introduction

We are all daily exposed to a number of pesticides from the food we eat and the water we drink. In food anlysed by The Danish Veterinary and Food Administration pesticide residues are often found. In 2001 pesticide recidues were found in aproximately 30% of the test samples taken from fruit produced in Denmark and in aproximately 70% of test samples taken from fruit produced outside Denmark (8). Pesticide residues are also found in vegetables and food crops but at lower levels (8).

The possible adverse health effects of a pesticide have traditionally been evaluated on the basis of the individual pesticide. However, even though we often are exposed to more than one pesticide through the food, the knowlegde of adverse health effects of combination of pesticides is only limited. It has been known for many years that pharmaceutical drugs can interact and therefore it is likely that other compounds like pesticides also interact. However, the levels for intake of pesticide residues is much lower than the therapeutical levels of a drug and interaction occuring at high dose levels may not be representative for low dose level exposure (11;16).

Various approaches have been suggested for risk assessment of mixtures of chemicals (14;20;21), but there are no internationally accepted procedures. It has been suggested that the most practical approach for evaluation of combined effects would be the assumption of an additive effect, since most compounds in combination have not more than an additive effect (14). The Danish Veterinary and Food Administration uses as a first cut-of an approach of summing all pesticide residues and compare the intake based on the acceptable daily intake (ADI) for the most toxic pesticide involved for the risk assessment (25). If the summarized ADI for all the pesticides exceeds the most toxic compound's ADI a Hazard-Index is calculated (8). A Hazard-Index is calculated as the sum of the theoretical intake of the individual pesticide in the food item in relation to the individual pesticides ADI (8).

The overall objectives of the animal experiments presented in this report was to contribute to the limited knowledge of risk assesments for intake of combination of pesticides.

2 Background – initial study

2.1 Objectives

In 1998/1999 a study was initiated with the objective of studying a possible combination effect of simultaneous exposure to five pesticides alphacypermethrin, bromopropylate, carbendazim, chlorpyrifos and mancozeb in rats. These pesticides were chosen as representative of pesticides occurring together in food produced in or imported into Denmark (for detailed results, see Appendix A)

2.2 Material and methods

The initial study was performed as a 28 days oral toxicity study. A combination of the 5 pesticides was administered to the rats in their diet for 28 days in four dose groups with 6 males and 6 females in each dose group. The dose levels were based on literature data and were set to 1/5 times NOAEL (low-dose), NOAEL (mid-dose), and 5 times NOAEL (high dose) of each pesticide, a control group was also included. A number of organs were examined by macroscopic and microscopic pathology, biochemical blood analyses were performed, and acetylcholinesterase activity in plasma and brain was measured.

2.3 Results

After one day of exposure via the test diet, the rats in the highest dose group showed severe toxic effects in the form of changed behaviour and ataxia indicating central nervous system disorder. Four rats were euthanized, while the remaining rats were put on control diet for two days, during which all toxic signs disappeared. The high dose group was terminated at this point. At sacrifice after 28 days' dosing, the rats in the mid-dose group appeared more active and sensitive to sound stimuli. There were no treatment-related differences in weight gain. The most marked biochemical effect was statistically significant acetylcholinesterase inhibition of the brain of 8% in low- and 12% in the mid-dose groups in female rats. Plasma acetylcholinesterase was decreased in females and most pronounced in the mid-dose group. A decreased WBC was found in males in the low- and mid-dose group. Differential count revealed a higher number of neutrophiles in the mid-dose group. In females this tendency was also found, but statistical significance was not reached. The only effect on organ weight was an increased absolute and relative liver weight in both sexes in the mid-dose group.

2.4 Discussion and conclusions

The high level of toxicity in the initial study was unexpected. Since a specific effect in the form of acetylcholinesterase inhibition was identified in low- and mid-dose groups, interest was focused on chlorpyrifos, the only acetylcholinesterase inhibitor among the five pesticides in the combination. The result could be explained as a true combination effect. However, since the dose of chlorpyrifos had been set on the basis of literature data, the

possibility that chlorpyrifos had been administered at effective levels above those intended could not be excluded / the NOAEL had to be reaffirmed under the conditions in our laboratory

A possible way, in which other compounds can enhance the toxic effect of chlorpyrifos, is by increasing the bio activation of chlorpyrifos to the active metabolite chlorpyrifos-oxon. This can occur e.g. by the other compounds causing an increase in the metabolic activity of the liver, thereby causing an increased production of the oxon. Another explanation could be that one or more of the other compounds interfere with the metabolism of the oxon resulting in a decreased rate of removal of the oxon.

The observed symptoms were signs of an acute toxic effect indicating changed CNS function. The two pesticides in the mixture that have an effect on CNS are chlorpyrifos and alphacypermethrin. However, as acetylcholinesterase inhibition was the critical effect in the study, further studies focused on a possible enhanced effect of chlorpyrifos caused by one or more of the other four pesticides.

It was therefore decided to conduct a series of two experiments.

A dose-response study for chlorpyrifos to clarify whether the literature-based dose used in the initial study was indeed a NOAEL.

Secondly, another combination study to try and confirm the previous findings, and to show a dose-response for inhibition of brain acetylcholinesterase by increasing the dose of chlorpyrifos while keeping a constant dose of the other four pesticides (alphacypermethrin, bromopropylate, carbendazim and mancozeb). Furthermore the study should provide data for explanation of the mode of interaction with focus on liver metabolism.

3 The five pesticides mode of action, target organ and possible interactions

Below, metabolism and target organs are described for the five pesticides alphacypermethrin, bromopropylate, carbendazim, chlorpyrifos and mancozeb. Based upon these data, the possible combined effects of the exposure to a mixture of the five pesticides are discussed.

3.1 Alphacypermethrin

Alphacypermethrin is a synthetic pyrethroid insecticide. Alphacypermethrin is rapidly absorbed and excreted via urine and faeces. Alphacypermethrin is metabolised by ester cleavage to give cyclopropanecarboxylic acid and the 3-phenoxybenzene moiety, which is excreted as the sulphate conjugate of 3-(4'hydroxyphenoxy) benzoic acid (7). Cypermethrin is a non-systemic insecticide with contact and stomach action (32). Alphacypermethrin is more biologically active than cypermethrin.

Alphacypermethrin is a neurotoxic compound interacting with the sodium channels in the peripheral and central nervous system (15). Alphacypermethrin causes a long-lasting prolongation of the normally transient increase in sodium permeability of nerve membrane during excitation, resulting in long-lasting trains of repetitive firing.(5). Signs of intoxication with alphacypermethrin in 28 and 90 day oral toxicity studies in rats at dose levels of 40 mg/kg bw and above were salivation, high-stepping and splayed gait, hunched posture, hypersensitivity to stimuli, reduced body weight and food consumption (5). In 90 days oral toxicity study in rats increase in relative liver weight, decrease in haemoglobin (males), decrease in mean corpuscular volume (MCV, males) and decrease in eosinophils (males) was seen (37).

3.2 Bromopropylate

Bromopropylate is an acaricide of the class benzilates. Bromopropylate is metabolised into 8 distinct metabolites, these are excreted in faeces, urine and expired air. There is a pronounced sex difference in metabolite pattern in urine where benzilic acid is a minor fraction in males (10-14%) and a major fraction in females (65-72%). Males excrete 90% trough faeces and 6% in urine, whereas females excrete 55% through faeces and 33% in the urine, a small amount is excreted through CO_2 expiration (35).

Bromopropylate induces liver enzymes such as cytochrome-P450, ethoxycoumarin O-deethylase, ethoxyresorufin O-deethylase, pentoxyresorufin O-depentylases, styrene oxide hydrolase's, cytosol gluthathione S-transferase, UDP-glucoronyl-transferase and testosterone hydrolase. This causes increase in liver weights at high dose levels as a result of functional overload, this is regarded a reversible adaptation (35).

The critical effects seen in 28 and 90 days oral toxicity studies are reduced body weight, increased liver, kidney and testis weight. In long term oral toxicity studies liver and kidney weights were increased, in addition the weight of the testis and the thyroid gland were increased in male rats. The histological changes in the testis were testicular tubular atrophy and oedema. In the thyroid gland the histological changes were follicular cystic dilatation and hyperplasia (35).

3.3 Carbendazim

Carbendazim is a fungicide of the class benzimidazoles. Carbendazim is readily absorbed after oral exposure in laboratory animals and rapidly metabolised. Carbendazim is metabolised to 5-HBC-S (main metabolite in males rats), 5,6-HOBC-Noxide (main metabolite in female rats), 5,6-DHBC-S and 5,6-DHBC-G the two minor metabolites. Carbendazim is excreted in faeces (25% male and 33-38% in females) and urine (36).

Relative liver weight increased in rats fed above 2000 ppm. Feeding carbendazim to rats at 2000 ppm or more resulted in slight-to-moderate induction of several phase-I drug metabolising enzymes: 7-ethoxycoumarin-O-deethylase, biphenyl-4-hydroxylase, aniline hydroxylase, 4-methoxybiphenyl-N-demethylase and cytochrome-c-reductase. The activities of the phase-II drug enzymes glucoronyl transferase I and II and the gluthathione content were moderately-to-markedly increased at this dose (36).

In 90 days oral toxicity studies in rats an increased liver weight, decreased RBC, decreased WBC, increased Alkaline Phosphatase (ALP) in male, decreased blood urea (males) and increased bilirubin were seen. Histological kidney damage with tubular dilatation and hydropic degeneration at 16 mg/kg, fibrosis and congestion at 32 and 64 mg/kg were seen. In a 90 oral toxicity study in dogs increase in liver and thyroid weight and decrease in heart weight was observed (36). Clinical signs of intoxication after a high single dose are generally non-specific. Tetisticular degeneration has been observed after high single doses (>1000 mg/kg bw) (36).

3.4 Chlorpyrifos

Chlorpyrifos [O, O,-diethyl O-(3,5,6-thrichloro-2pyridinyl) phosphorothiate] is a broad-spectrum organ phosphorus pesticide used as an insecticide. Chlorpyrifos is bio activated by the microsomal cytochrome-P450 system to the active oxon metabolite, which is about three times more potent as an acetylcholinesterase inhibitor than chlorpyrifos itself (18). Most bio activation takes place in the liver, while detoxification takes place in the liver and plasma. The degradation step occurs by conversion directly to 3,5,6-trichloro-2-pyridinol (TCP) and diethyl thiophosphate. The oxon can be deactivated by hydrolysis to diethylphosphate and TCP (18;29). A minor reaction pathway is hydrolysis to monethyl 3,5,6-trichloro-2-pyridyl phosphorothionate. The oxon is further metabolised by hydrolysis catalysed by paraoxonase.

Acute toxicity of chlorpyrifos is caused by acetylcholinesterase inhibition in CNS. Acetylcholine is the neurotransmitter of the parasympatic nervous system. Acetylcholinesterase is the enzyme that cleaves acetylcholin in to acetate and choline. An inhibition of acetylcholinesterase increases the amount of acetylcholin available for the parasympatic nervous system and signs of intoxication are therefore stimulation of the parasympatic nervous system and include salivation, dyspnoea, flaccid paralysis, vomiting, piloerection, exophtalmia and diarrhoea. Female animals are generally more sensitive to acute effects of chlorpyrifos than male animals (38).

The critical effects in 28 and 90 days oral toxicity studies in rats are decreased body weights (bw), increased vacuolation of the adrenal zona fasciculata, decrease in red blood cell count (RBC), increase in platelet count (Plt), reduced serum total protein, albumin and globulin concentrations, decrease in alanine aminotransferase (ALAT) and ALP activity in males, decrease serum glucose concentration and increase in specific urine gravity in females. Other targets are acetylcholinesterase inhibition in plasma, erythrocyte and brain (38).

3.5 Mancozeb

Mancozeb is a dithiocarbamate used as fungicide. Mancozeb is rapidly absorbed, metabolised and excreted in both sexes in rats. Mancozeb is metabolised to ethylene thiourea (ETU), ethylenethiuram monosulfide, EBIS, ethylenethiourea-N-thiocarbamid N-acetyl-ethylenediamine, ethylenediamine, ethylene urea, creatine, allantoin plus six unknown metabolites. ETU is the major metabolite. Mancozeb is distributed to various organs with the highest concentrations in thyroid tissue (35).

In 28 and 90 days oral toxicity studies in rats at 1000 ppm increased liver and thyroid weights and decreased body weights were seen. In female rats increased spleen weights were seen at dose levels of 1000 ppm. Serum Thyroxin (T_4) was decreased in rats at 1000 ppm, TSH increased in rats at 250 ppm and plasma cholinesterase increased at 2576 ppm (35).

In rats histological changes were thyroid follicular cell hyperplasia in 90% of the rats receiving 1000 ppm, in the kidney a yellow-brow pigment in the lumen of the cortical tubules was seen of rats at dose levels above 125 ppm, in the adrenal gland hypertrophy of cells in zona glomerulosa was seen at dose level 1000 ppm and in the liver centrilobular cell hypertrophia was seen in males receiving 1000 ppm (35).

In dogs a decrease in thymus weight and an increase in thyroid weight at dose levels at 1000 ppm or above was seen. By microscopy this was recognized as thymic cortical depletion and thyroid follicular cell hyperplasia. In the blood a reduction in RBC, haemoglobin and haematocrit was seen accompanied by increase in mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) at dose levels of 1000 ppm for females and 5000 ppm for males. In another dog study females showed frank anaemia at dose levels of 113 mg/kg bw/ day and a dose-related decrease in RBC. In blood chemistry T_3, T_4 and ALAT was decreased at 5000 ppm, and cholesterol and bilirubin was increased for females at dose levels at 1000 ppm and in males at dose levels of 5000 ppm (35). Long-term animal studies have shown mancozeb to be a multi-potent carcinogen causing tumours in mammae, zimbal gland,

liver, pancreas, thyroid gland, bone and blood system (2). The metabolite ETU of mancozeb is also a thyroid carcinogen (28).

3.6 Possible interaction between the five pesticides

Main critical effects and ADI for the 5 pesticides are presented in Table 1.

The liver is target organ for four of the five pesticides, these are alphacypermethrin, bromopropylate, carbendazim and mancozeb. Carbendazim and bromopropylate induce liver enzymes like cytochrome-P450 and ethoxycoumarin O-deethylase. It is therefore likely that a combination of all or some of the pesticides will cause increased liver weight and induce liver enzymes. Induction of liver enzymes might increase the metabolism of some of the other pesticides, which might increase or decrease their toxicity.

The kidneys, testis and the thyroid gland are organs commonly affected by bromopropylate, carbendazim and mancozeb. Reduced/increased weights of these organs might be expected as a combination effect.

Blood is affected by chlorpyrifos, carbendazim and mancozeb. RBC is reduced at high doses of chlorpyrifos, carbendazim and mancozeb. A reduction in RBC might be expected as a possible result of interaction between the pesticides.

CNS is the target organ of two of the pesticides chlorpyrifos and alphacypermethrin. Chlorpyrifos acts by inhibiting acetylcholinesterase and signs of intoxication are stimulation of the parasympatic nervous system causing salivation, dyspnoea, flaccid paralysis, vomiting, piloerection, exophthalmia and diarrhoea (38). Alphacypermethrin acts by changing sodium channels by increasing the permeability of sodium in nerve membranes during excitation, resulting in long-lasting trains of repetitive firing. Signs of intoxication with alphacypermethrin are salivation, high-stepping and splayed gait, hunched posture, hypersensitivity to stimuli, reduced body weight and food consumption (5). Thus both alphacypermethrin and chlorpyrifos interfere with the function of the nervous system, however, resulting in different clinical signs.

At dose-levels of NOAEL none of the compounds are expected to have an effect by themselves. However, there might be toxicological effect of the mixture due to interaction of the individual compounds when these chemicals are given simultaneously at dose levels of NOAEL. Expected target organs for combined effects of the five pesticides in the mixture are CNS, blood, liver, kidney, testis and thyroid gland.

Table 1. Critical effects and Acceptable-Daily-intake (ADI) in rats or dogs of the five pesticides chlorpyrifos, alphacypermethrin, bromopropylate, carbendazim and mancozeb. ADI is not always based on 28 and 90 oral toxicity studies.

Pesticide	Critical effects	ADI	Reference
Chlorpyrifos	Nervous system, adrenal gland, blood (RBC ↓,Plt ↑)	0.01 mg/kg bw/day	(38)
Alphacypermethrin	Central and peripheral nervous system, liver, blood (Hb↓, MCV↓)	0.05 mg/kg bw/day	(7) (32) (15)
Bromopropylate	Liver, kidney, thyroid (males), testis, body weight	0.03 mg/kg bw/day	(35)
Carbendazim	Liver, kidney, testis, epididymis, lung, blood (RBC↓, WBC↓)	0.03 mg/kg bw/day	(36)
Mancozeb	Liver, kidney, spleen, thyroid, thymus, body weight, blood (RBC↓, hb↓, hct↓)	0.03 mg/kg bw/day	(35)

4 Dose-response study of Chlorpyrifos

4.1 Objectives of the dose-response study of chlorpyrifos

The objectives of this study was to characterise the dose-response of chlorpyrifos and compare results with the NOAEL set by WHO based on the scientific literature.

4.2 Material and methods

The study design followed OECD guideline 407 'repeated dose 28 day oral toxicity study' with minor deviations.

4.2.1 Animals

Male and female rats, 24 of each sex, of the Brl:WIST Han@Mol strain were obtained from M&B A/S, Ry, Denmark. The animals were 4 weeks of age on arrival (on 29 March 2001). Groups of six animals per sex were formed by stratified randomisation. The group mean body weight was within 5 % of the overall mean for each sex. As up to 10% is acceptable, this is regarded as satisfactory. Males weighed between 44 and 66 g (overall mean 55.2 g), females weighed between 52 and 72 g (overall mean 61.6 g). The animals were approximately 5 weeks old at the start of dosing on 5 April 2001.

4.2.2 Environment

The animals were housed in a single, exclusive room, air-conditioned to provide a minimum of 10 air changes per hour. The temperature in the animal experimental room was 22±1°C, and the relative humidity was 55%±5%. Fluorescent lighting was controlled automatically to give a cycle of 12 hours light (0900 to 2100) and 12 hours dark. The animals were housed in pairs by sex in macrolon cages type III with Tapvei aspen wood bedding.

4.2.3 Diet and water

Diet was provided ad libitum. All rats received control group diet for 6 days until the start of the dose period. During the dose period, the rats received test diet according to dose group. Acidified tap water was provided ad libitum in nipple bottles (citric acid, pH=3). Drinking water was from the municipal water supply.

4.2.4 Test and control substances

The test substance, a brown solid, was chlorpyrifos, CAS No. 002921-88-2 supplied by Dow Chemicals. The purity of the test substance was 98.6%. The control substance and vehicle for the test substance was acetone. The test substance was administered orally in the diet. The animals received the substance in the diet for 28 days excluding the day of sacrifice.

4.2.4.1 Dose levels

In Table 2 the selected dose levels are shown.

Table 2. Group number, group description in words and dose levels in ppm and mg/kg/day for amount of chlorpyrifos (CH) given to each dose group, and number of animals per group (male/female).

Group Number	Description	Dose level (ppm in food)	Equivalent dose level (mg/kg/day)1	Animals/group (male)	Animals/group (female)
1	Control	0 (vehicle only)	0	6	6
2	Low, 1/25 x NOAEL CH	0.06	0.006	6	6
3	Intermediate, 1/5 x NOAEL CH	0.3	0.03	6	6
4	High, 1 x NOAEL CH	1.5	0.15	6	6

4.2.4.2 Test substance formulation and preparation

Four test diets with added vehicle (acetone) and/or chlorpyrifos (0.06, 0.3, 1.5 mg/kg diet) were prepared by Altromin International, Germany. Diets were prepared once for the entire study. The test substance was added to powdered diet and made to pellets. The diets were stored in the original bags at room temperature.

4.2.4.3 Stability and homogeneity

From the initial study (see Appendix A) it was known that chlorpyrifos is stable for at least 28 days. In the present study, the concentration in week 4 was determined (see Table 3). The test diets were not analysed for homogeneity. Samples of 50 g of each test diet were obtained during week 4 of the dosing period. Each sample was analysed in duplicates. The achieved concentrations are shown in Table 3. The concentrations of pesticides in the diets were regarded as satisfactory.

Table 3. Intended and measured concentration of chlorpyrifos in the diet for each dose group (see Table 2).

Dose group	Intended concentration (mg/kg feed)	Measured average concentration (mg/kg feed)
Group 1	0	not found
Group 2	0.06	0.083
Group 3	0.3	0.35
Group 4	1.5	1.32

4.2.5 Pre-test procedures

All animals were given a health inspection at arrival and observed daily during the 7-day acclimatisation period. The animals were assigned to treatment groups during the acclimatisation period using a randomisation procedure based on body weight.

[1] based on OECD guidance notes for analysis and evaluation of chronic toxicity studies 2000(24)

Group body weight means and standard deviations were calculated and inspected to ensure there were no unacceptable differences between groups. The animals were individually identified by ear clipping (see Table 4). Cages were appropriately identified/ tagged with study information including study number and animal numbers.

Table 4. Group number, group colour code and animal identity numbers (ear clipping) for male and female rats.

Group Number	Group codes	Animals/group (male)	Animals/group (female)
1	White	1-6	7-12
2	Blue	13-18	19-24
3	Green	25-30	31-36
4	Yellow	37-42	43-48

4.2.6 Experimental procedures

4.2.6.1 Clinical signs, morbidity and mortality

All animals were observed twice daily in their cage for signs of adverse health or overt toxicity. A record was maintained of the clinical conditions for the study population as a whole. If adverse clinical signs were observed, an individual record was kept for the animals concerned. However, no adverse clinical signs were observed during the study.

4.2.6.2 Body weights

Individual body weights were recorded at weekly intervals. The last of these measurements was done in the morning on the day of sacrifice, and these body weights were used for calculation of relative organ weights.

4.2.6.3 Food and water consumption

The amount of food and water consumed by the animals in each cage was measured weekly.

4.2.6.4 Functional observational battery (FOB)

All animals were subjected to a battery of behavioural tests and observations (as listed below) during the fourth week of the dose period. At the time of testing, the observer was unaware of each animal's dose level. Each animal was assigned a code number and moved to a new cage (1 animal/cage) by an independent person.

Observational measurements were performed before and upon removal from the home cage. The animal was placed into a square arena for 3 minutes. During this time, behaviour was evaluated as indicated (open field). Various manipulative tests were also done.

The following observations and measurements were done:
In the home cage: posture, eye opening, convulsions, and vocalisation.
During removal from the cage: reaction to handling, piloerection, lacrimation, salivation and eye opening.
In the open field arena: number of rears during 3 minutes, convulsions, gait, ease of movement, arousal, number of fecal boli and urine pools during 3 minutes.

Reflex testing: reaction to an object moved towards the face, to a push to the hindquarter, to a sudden noise, to a tail pinch, and the pupillary response to light.
Assessment of righting reflex, foot splay, grip strength of fore- and hind limbs. Measurement of body weight and temperature. Any other observations were recorded.

4.2.6.5 Motor activity measurement
Following FOB testing, the motor activity of each animal was assessed in an automated photocell activity recorder for 30 minutes. Activity counts were recorded in 5-minute intervals. Thus, data were obtained from six 5-minute motor activity intervals, called periods.

4.2.6.6 Eye examinations
Eye examinations were performed on all rats following FOB testing, before blood sampling. The assessment included pupillary response to light, and examination of the eye by direct observation, and following drug-induced (tropicamide) pupillary dilatation examination with a slit lamp and by indirect ophthalmoscopy.

4.2.6.7 Plasma acetylcholinesterase activity
Blood samples (1.0 ml intended) were drawn from all animals on the day before sacrifice. Samples were collected from the orbital sinus under CO_2/O_2 anaesthesia. Blood was collected in heparinized tubes and analysed for acetylcholinesterase activity. Samples were analysed on the day of sacrifice or refrigerated and analysed the following day. The analysis was performed at 37 °C on a Cobas Mira auto analyser by use of a commercially available kit obtained from Boehringer Manheim applying Calibrator® Roche as the calibrator.

4.2.6.8 Brain acetylcholinesterase activity and protein concentration
At sacrifice the brain was removed and separated sagittally into two halves. The left half was used for measurement of brain acetylcholinesterase activity. The tissue was homogenized. A 2 ml sample of the homogenate was analysed for acetylcholinesterase activity and protein content. Relative brain acetylcholinesterase activity was calculated (units/g protein). Samples were analysed on the day of sacrifice or refrigerated and analysed the following day. The analysis was performed at 37 °C on a Cobas Mira auto analyser by use of a commercially available kit obtained from Boehringer Manheim applying Calibrator® Roche as the calibrator. The protein analysis was performed on a Hitachi 912 auto analyser by use of a commercially available kit obtained from Roche applying CFAS® Roche as the calibrator.

4.2.7 Terminal procedures

4.2.7.1 Necropsy and macroscopic pathology
All animals were subjected to necropsy. The order of necropsy was balanced with respect to dose group and sex. The animals were euthanized by decapitation in CO_2/O_2 anaesthesia (on 3 May 2001). A full macroscopic examination was performed and all lesions were recorded.

4.2.7.2 Organ weights
The following organs of all animals were weighed: liver, kidneys, adrenal glands, testes, thymus and brain. The last of the weekly body weight

measurements, obtained on the day of sacrifice, was used for calculation of relative organ weights.

4.2.7.3 Histology

The following tissues were preserved in formaldehyde: the above-mentioned organs (from the brain the right half only) and in addition tissues with macroscopic lesions. It was planned to perform histopathology on the brain and on organs with macroscopic lesions and on organs with statistically significant deviation in relative weight.

4.2.7.4 Brain histology

Histological examination of the brains of group 1 and 4 was performed on slides stained with haematoxylin and eosin (HE). Brain tissue was examined for glial fibrillary acidic protein (GFAP) content by immunocytochemistry, using antibodies to the glial filament. GFAP staining is a commonly used method to examine the distribution of astrocytes and their hypertrophic response to neurotoxicants. The method allows for the direct visualization of astrocytes. The brains from control animals and group 4 were stained for GFAP and evaluated under light microscopy for expression of GFAP and distribution of astrocytes.

4.2.8 Data evaluation

Data from treated animals were compared with control data. Males and females were evaluated separately.

No statistical analysis was done on data where it was obvious that no group difference was present (some FOB data, eye examination data[2], brain GFAP content).

Continuous data were evaluated with respect to conformity with normal distribution and variance homogeneity, and it was determined whether a parametric or a non-parametric test would be more suitable.

Two-way repeated measures analysis of variance was used for: body weight, relative food- and water consumption, and motor activity data.

Two-way analysis of variance was used for: organ weights, continuous FOB data. The analysis was ANOVA with Dunnett's test for pair wise comparisons.

Non-parametric analysis of variance was used for: Acetylcholinesterase activity. The analysis was Kruskal-Wallis with Wilcoxon rank sum test for pair wise comparisons.

[2] only data for visual inspection and pupillary response to light were collected from all animals. Data for slit lamp and ophthalmoscopy were not collected from 7 males and 10 females, and therefore these two endpoints were not considered for statistical analysis.

4.3 Results

4.3.1 Clinical signs, morbidity and mortality

No adverse clinical signs were observed, and no animals died during the study.

4.3.2 Body weights

All groups gained weight during the study period (Statistically significant effect of time). There was no statistically significant difference among groups within each sex.

4.3.3 Relative food and water consumption

All groups showed the normal decrease in relative food and water consumption during the study period (statistically significant effect of time). There was no statistically significant difference among groups within each sex. The relative food and water consumption (g food or water/kg bodyweight/day) in the control and high dose groups are shown in Table 5 and 6.

Table 5. Relative food consumption (g food /kg bodyweight/day) in week 1 and week 4 for females and males in control (group 1) and high dose (group 4) groups. See dose groups in Table 2.

	Week 1	Week 4
Females, Control	153±11	96±7
Females, High dose	161±12	103±7
Males, Control	168±19	96±7
Males, High dose	169±8	106±7

Table 6. Relative water consumption (g water /kg bodyweight/day) in week 1 and week 4 for females and males in control (group 1) and high dose (group 4) groups. See dose groups in Table 2.

	Week 1	Week 4
Females, Control	179±16	157±36
Females, High dose	202±17	180±15
Males, Control	189±21	124±10
Males, High dose	206±12	147±13

4.3.4 Functional observational battery (FOB)

No unusual behaviour was observed. The only statistically significant difference was a 16% reduced forelimb grip strength in females of the high dose group (1.5 ppm, group 4). No dose response was seen. However, it cannot be excluded that the reduced forelimb grip strength in females is substance related, since this was only seen in the high dose group.

4.3.5 Motor activity measurements

The variability in the data was large, which is commonly observed. Total motor activity during 30 minutes was unaffected by treatment. In all groups, a decline in activity over time was found reflecting the habituation to the novel situation. This decline was statistically significant. There was no difference between treatment groups in the repeated measures analysis of individual 5-minute activity periods.

4.3.6 Eye examinations

Because of technical failure of the eye examination utensils, an overall diagnosis could not be made in 7 males (0, 3, 3 and 1 animal at 0, 0.06, 0.3 and 1.5 ppm respectively) and in 10 females (1, 2, 3 and 4 animals at 0, 0.06, 0.3 and 1.5 ppm respectively), however all eyes were examined by inspection and pupil response to light was assessed. No treatment-related abnormalities were observed. Overall, the eye examination is considered inconclusive due to the lacking slit lamp and ophthalmoscopy data.

4.3.7 Acetylcholinesterase activity

4.3.7.1 Plasma acetylcholinesterase activity

No treatment-related difference was found. The plasma acetylcholinesterase level was approximately four times higher in females compared to males (see Figure 1). This is a normal finding.

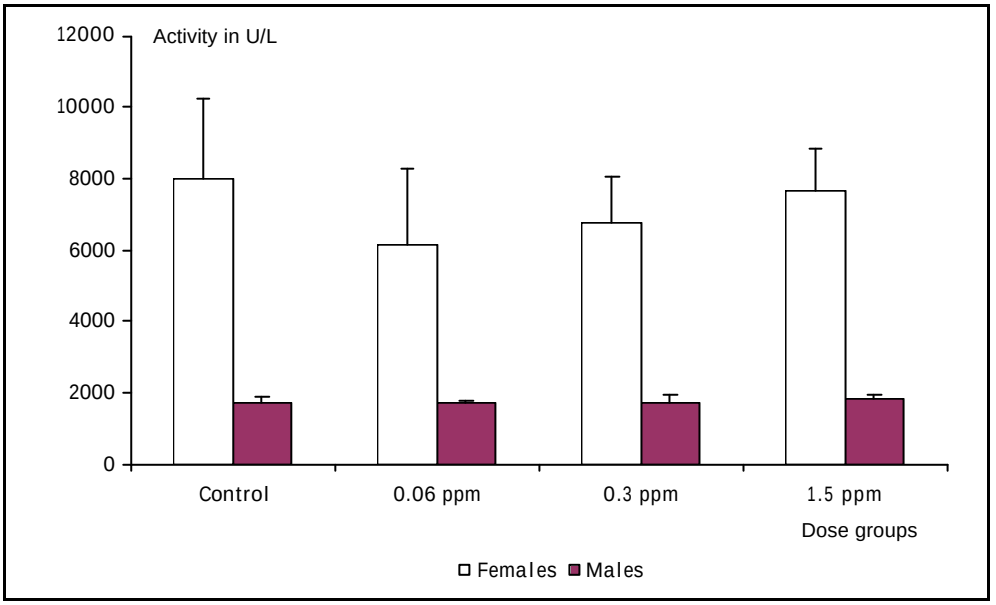

Figure 1. Plasma acetylcholinesterase activity, males and females for the 4 dose groups. Control (group 1), 0.06 ppm chlorpyrifos (group 2), 0.3 ppm chlorpyrifos (group 3) and 1.5 ppm chlorpyrifos (group 4).

4.3.7.2 Relative brain acetylcholinesterase activity

No treatment-related difference was found. The relative brain acetylcholinesterase level was the same in females and in males.

Acetylcholinesterase activities measured in plasma and brain in this study are erroneously about a factor 4 too high, because the calibration value for butyrylcholineesterase activity by mistake was programmed in the auto

analyser. Consequently, all values are about a constant 4 too high, but this does not influence the results of the study.

4.3.8 Pathology

4.3.8.1 Macroscopic pathology

At autopsy haemorrhages were found in the thymus of males and females in the groups 2, 3 and 4. These findings are normally related to euthanasia by CO_2/O_2 and decapitation. The few other macroscopic findings are considered spontaneous lesions unrelated to treatment.

4.3.8.2 Organ weights

No statistically significant treatment-related changes in absolute and relative organ weights were observed (group means of organ weights are presented in Appendix B)

4.3.8.3 Histopathology

As no organ weight changes were detected, no overall group histological examination was performed. One male rat in group 4 exhibited a changed bladder wall thickness (described grossly as muscular hypertrophy in the bladder), which was evaluated by microscopy to be a severe hyperplasia of the bladder epithelium.

4.3.8.4 Brain histology

No changes in colour intensity or distribution of astrocytes were seen in brains stained for GFAP in either control animals or in the high dose group (group 4). No histopathological findings were identified in brains stained with HE in either control animals or in group 4.

4.4 Discussion and conclusion of the dose-response study

The high level of toxicity in the initial study was unexpected, since a specific effect in the form of brain acetylcholinesterase inhibition was identified in all dose groups. The interest was focused on chlorpyrifos, the only acetylcholinesterase inhibitor among the five pesticides in the combination. The result could be explained as a true combination effect. However, since the dose of chlorpyrifos had been set on the basis of literature data, the possibility that chlorpyrifos had been administered at effective levels above those intended could not be excluded.

The objectives of the present study were to characterise the dose-response of chlorpyrifos and compare results with the NOAEL based on the scientific literature. Apart from a slight significant reduced forelimb grip strength in the high dose females, no effects were found in any dose group either on clinical signs, morbidity or mortality, body weights, food or water consumption, behaviour, pathology, plasma or brain acetylcholinesterase activity. Therefore it was concluded that the present study confirms the NOAEL of 1.5 ppm based on scientific literature. Furthermore the present study supports the hypothesis that the effects found in the initial study were related to the toxicity of the combination of the five pesticides and not to an unintended overdose of chlorpyrifos.

5 Combined exposure study

5.1 Hypothesis and objectives of the combined exposure study

It is hypothesised that the effect of chlorpyrifos on brain acetylcholinesterase inhibition is enhanced by the co-administration of the four pesticides: alphacypermethrin, bromopropylate, carbendazim and mancozeb.

The primary objectives of this study were first to confirm the effects identified in the initial study (increased brain acetylcholinesterase inhibition), and if possible to show a dose-response of brain acetylcholinesterase by varying the concentration of chlorpyrifos while keeping the concentration of the other four pesticides at a constant concentration. Second, to examine if liver metabolism of chlorpyrifos to the active metabolite chlorpyrifos-oxon was the mode of action.

5.2 Material and methods

The study design followed OECD guideline 407 'repeated dose 28 day oral toxicity study' with minor deviations.

5.2.1 Animals

Male and female rats, 96 of each sex, of the Brl:WIST Han@Mol strain were obtained from M&B A/S, Ry, Denmark. The animals were 4 weeks of age on arrival (on May 13, 2002). Groups of eight animals per sex were formed by stratified randomisation. The group mean body weight was within 5 % of the overall mean for each sex. Males weighed between 49 and 66 g (overall mean 55.0 g), females weighed between 48 and 67 g (overall mean 54.8 g). The animals were approximately 5 weeks old at the start of dosing (May 20, 2002).
The combined exposure study included two subsets of rats, each consisting of 6 groups with 8 rats of each sex, with a total 48 rats per sex. The two subsets of rats were given the same test diets (starting on May 20, 2002). One subset was sacrificed after 1 week of dosing (May 27, 2002), while the other subset was sacrificed after 4 weeks of dosing. The purpose of the first subset was to obtain 1-week blood samples for analysis of chlorpyrifos, chlorpyrifos metabolites, bromopropylate, carbendazim and alphacypermethrin. The first subset is referred as satellite study under plasma pesticide concentration. The second subset was the core study population on which all other examinations were done.

5.2.2 Environment

The animals were housed in a single, exclusive room, air-conditioned to provide a minimum of 10 air changes per hour. The temperature was 22±1°C, and the relative humidity was 55%±5%. Fluorescent lighting was controlled automatically to give a cycle of 12 hours light (0900 to 2100) and 12 hours dark. The animals were housed in pairs by sex in Macrolon cages type III with Tapvei aspenwood bedding.

5.2.3 Diet and water

Diet was provided ad libitum. All rats received standard diet (not containing vehicle) during the 7-day period from arrival to the start of dosing. During the dosing period, the rats received test diet according to dose group. Acidified tap water was provided ad libitum in nipple bottles (citric acid, pH=3). Drinking water was from the municipal water supply.

5.2.4 Test and control substances

The test substances, purity and supplier are shown in Table 7.

Table 7. Test substances names, purity and supplier

Substance name	Purity	Supplier (samples donated by the suppliers free of charge)
Chlorpyrifos	98.6%	Dow Chemicals
bromopropylate	94.1%	Syngenta Crop Protection Münchwilen AG, Im Breitenloh 180, CH-4333 Münchwilen, Switzerland
Alphacypermethrin	96.1%	BASF Aktiengesellschaft, Crop Protection Division, Agricultural Center, D-67114 Limburgerhof, Germany
Carbendazim	99.6%	BASF Aktiengesellschaft, Crop Protection Division, Agricultural Center, D-67114 Limburgerhof, Germany
Mancozeb	80-85%[a]	Rohm and Haas France S.A.S., La Tour de Lyon, 185 Rue de Bercy, F-75579 Paris, Cedex 12

a 15-20% related reaction products

The control substance and vehicle for the test substances was acetone. The test substances were administered orally in the diet. The animals received the substance in the diet for 28 days excluding the day of sacrifice.

5.2.4.1 Dose levels

The dose levels selected are shown in Table 8.

5.2.4.2 Test substance formulation and preparation

Altromin International, Germany, prepared the 6 test diet. Diets were prepared once for the entire study. The test substances according to 6 dose levels were dissolved in 50 ml of acetone and added to 1000 g of powdered diet altromin 1321, which was homogenised. The 1000 g were mixed and homogenised with another 4000 g of diet altromin 1321. The 5000 g were mixed and homogenised with another 20000 g of diet altromin 1321. The powdered diet was made in to pellets.

5.2.4.3 Storage

The diets were stored in the original bags at room temperature.

Table 8. Group number, group description and dose levels in ppm and mg/kg/day for amount of chlorpyrifos (CH), bromopropylate (BR), alphacypermethrin (AL), carbendazim (CB) and mancozeb (MA) given to each dose group, and number of animals (male/female)

Group Number	Description	Dose level (ppm in food)	Equivalent dose level (mg/kg/day)[3]	Animals/group (male)	Animals/group (female)
1	Control	0	0	16	16
2	Chlorpyrifos alone at NOAEL	CH:1.5	0.15	16	16
3	4 pesticides at NOAEL, CH at 1/25 x NOAEL	CH:0.06 BR:300 AL:180 CB:450 MA:125	0.006 30 18 45 12.5	16	16
4	4 pesticides at NOAEL, CH at 1/5 x NOAEL	CH:0.3 BR:300 AL:180 CB:450 MA:125	0.03 30 18 45 12.5	16	16
5	4 pesticides at NOAEL, CH at 1 x NOAEL	CH:1.5 BR:300 AL:180 CB:450 MA:125	0.15 30 18 45 12.5	16	16
6	4 pesticides at NOAEL, CH at 2 x NOAEL	CH:3.0 BR:300 AL:180 CB:450 MA:125	0.3 30 18 45 12.5	16	16

5.2.4.4 Stability and homogeneity

It was planned to take samples of 50 g of each test diet during week 4 of the dosing period. However, by a mistake the samples were taken (14 July 2002) weeks later. Analyses for alphacypermethrin, bromopropylate, chlorpyrifos, and carbendazim were performed at the Institute of Food Safety and Nutrition. Analysis for mancozeb was done at Food Region Copenhagen. Confirmation analysis of mancozeb was performed by the Pesticide laboratory for analysis of pesticides in fruit, vegetables, water and soil at the Plant Protection Centre, Ås, Norway. All laboratories are accredited.
Each sample was analysed twice. The measured concentrations (mean of 2 replicates) and percentages of the intended concentration are shown in Table 9. The concentration of mancozeb was clearly below the intended concentration, and the concentration of chlorpyrifos in group 3 was three times the intended. The pesticide concentrations in the feed are therefore not regarded as satisfactory in relation to the intended.

To examine homogeneity five samples were taken from the test diet for group 3. The five samples were taken from various places in the bag. The results (mean of 2 replicates) of the analysis are shown in Table 10. The homogeneity is regarded as satisfactory.

[3] based on OECD guidance notes for analysis and evaluation of chronic toxicity studies 2000(24)

Table 9. The measured concentration of pesticides in the diet (mg/kg)/ and the percentages of the intended concentrations for the six dose groups. Chlorpyrifos (CH), bromopropylate (BR), alphacypermethrin (AL), carbendazim (CB) and mancozeb (MA). See dose groups in table 8.

	Group 1 mg/kg	Group 2 mg/kg / %	Group 3 mg/kg / %	Group 4 mg/kg / %	Group 5 mg/kg / %	Group 6 mg/kg / %
CH	<0.05	1.56/104%	0.20/339%	0.40/135%	1.54/103%	2.41/80%
BR	<0.05	<0.05	190/63%	224/75%	237/79%	241/80%
AL	<0.05	<0.05	123/68%	131/73%	149/83%	153/85%
CB	<0.05	<0.05	409/91%	349/78%	375/83%	380/84%
MA	<0.5	<0.5	22/18%	27/22%	30/24%	32/26%

Table 10. Measured concentrations (mg/kg) of pesticides Chlorpyrifos (CH), bromopropylate (BR), alphacypermethrin (AL), carbendazim (CB) and mancozeb (MA) in the diet of dose group 3. Samples are taken from various places in the bag of the diet for group 3.

	Sample 1 mg/kg	Sample 2 mg/kg	Sample 3 mg/kg	Sample 4 mg/kg	Sample 5 mg/kg	Mean ± STD mg/kg
CH	0.22	0.17	0.19	0.23	0.23	0.21±0.03
BR	190	233	217	243	224	221±20
AL	123	123	143	155	150	139±15
CB	409	359	379	342	419	381±33
MA	22	27	21	28	27	25±3

5.2.5 Pre-test procedures

All animals were given a health inspection on arrival and observed daily during the 7-day acclimatisation period. The animals were assigned to treatment groups during the acclimatisation period using a randomisation procedure based on body weight. Group body weight means and standard deviations were calculated and inspected to ensure there were no unacceptable differences between groups. The animals were individually identified by ear clipping (see Table 11).

Table 11. Ear clip animal identity number for males and females in the 6 dose groups

Group number	Animals/group (male)	Animals/group (female)
1	1-16	17-32
2	33-48	49-64
3	65-80	81-96
4	97-112	113-128
5	129-144	145-160
6	161-176	177-192

Cages were appropriately identified with study information including study number and animal numbers.

5.2.6 Experimental procedures

5.2.6.1 Clinical signs, morbidity and mortality

All animals were observed twice daily in their cage for signs of adverse health or overt toxicity. A record was maintained of the clinical conditions for the

study population as a whole. If adverse clinical signs were observed, an individual record was to be kept for the animals concerned.

5.2.6.2 Body weights

Individual body weights were recorded at weekly intervals. The last of these measurements was done in the morning on the first day of sacrifice (June 17, 2002), and these body weights were used for calculation of relative organ weights.

5.2.6.3 Food and water consumption

The amount of food and water consumed by the animals in each cage was measured weekly.

5.2.6.4 Eye examinations

Eye examinations were performed during the acclimatisation period on all rats scheduled for sacrifice after 4 weeks. A second examination was performed in the fourth test week. The week 4 examination included rats in group 1 and 6 (vehicle control and highest dose group), while the intermediate groups were only to be included if an increased incidence of eye abnormalities were found in group 6. As this was not the case, groups 2-5 were not examined at the examination in the fourth week. The assessment included evaluation of pupil response to light, and examination of the eye by direct observation. Subsequently, pupillary dilatation was achieved by application of 1 drop of 0.5% tropicamide applied to each eye 5-15 minutes prior to examination, allowing examination of the superficial and deeper eye structures with slit lamp and by indirect ophthalmoscopy.

5.2.6.5 Functional observational battery (FOB)

All animals were subjected to a battery of behavioural tests and observations (as listed below) during the fourth week of the dose period.
At the time of testing, the observer was unaware of each animal's dose level. Each animal was assigned a code number and moved to a new cage (1 animal/cage) by an independent person.

Observational measurements were performed before and upon removal from the home cage. The animal was placed into a square arena for 3 minutes. During this time, behaviour was evaluated as indicated (open field). Various manipulative tests were also done.

The following observations and measurements were done:
In the home cage: posture, eye opening, convulsions, and vocalisation.
During removal from the cage: reaction to handling, piloerection, lacrimation, salivation and eye opening.
In the open field arena: number of rears during 3 minutes, convulsions, gait, ease of movement, arousal, number of faecal boli and urine pools during 3 minutes.
Reflex testing: reaction to an object moved towards the face, to a push to the hindquarter, to a sudden noise, to a tail pinch, and the pupil response to light. Righting reflex, foot splay, grip strength of fore- and hind limbs, body weight and temperature. Any other observations of behaviour or appearance made during testing were noted.

5.2.6.6 Motor activity measurement

Following FOB testing, the motor activity of each animal was assessed in an automated photocell activity recorder for 30 minutes. Activity counts were

recorded in 5-minute intervals. Thus, data were obtained from six 5-minute motor activity intervals, called periods.

5.2.6.7 Haematology

Blood samples (0.5 ml intended) were drawn from all animals the week before sacrifice. The rats were anaesthetized with 0.2 ml/100 g of a solution of Hypnorm®/Dormicum®[4] s.c., and the blood was collected from the tail vein. WBC, RBC, Hgb, HCT, MCV, MCH, MCHC, Plt was measured on an animal blood counter, Pentra 120 (Triolab, Brøndby, Denmark). Differential blood cell count was made under light microscope on blood smears on glass slides stained with May-Grünwald colour (Merck 1424). Differential counts were made as duplicates for each animal in groups 1 and 6.

5.2.6.8 Plasma acetylcholinesterase activity

Blood samples (as much as possible, 4-5 ml intended) were drawn from all animals on the day of sacrifice. The rats were anaesthetized with Hypnorm®/Dormicum® as described under haematology, and the blood was collected from the heart. For analysis of plasma acetylcholinesterase activity 1 ml of blood was collected in heparinized tubes and analysed for acetylcholinesterase activity. Samples were analysed on the day of sacrifice or refrigerated and analysed the following day. Principally, the analysis is a minor modification of the method originally described by Ellman et al.(4). The analysis was performed at 37 °C on a Hitachi 912 auto analyser applying CFAS® Roche as the calibrator.

5.2.6.9 Brain acetylcholinesterase activity and protein concentration

At sacrifice the brain was removed and separated sagittally into two halves. The left half was used for measurement of brain acetylcholinesterase activity. The tissue was homogenized, and a 2 ml sample of the homogenate was analysed for acetylcholinesterase activity and protein content. Relative brain acetylcholinesterase activity was calculated (units/g protein). Samples were analysed on the day of sacrifice or refrigerated and analysed the following day. The protein analysis was performed on a Hitachi 912 auto analyser by use of a commercially available kit obtained from Roche applying CFAS® Roche as the calibrator.

5.2.7 Plasma pesticide concentrations

Method for analysis of rat plasma for alphacypermethrin, bromopropylate, carbendazim, chlorpyrifos and its degradation products chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol (TCP) are described below. We did not get enough plasma from the rats to develop and validate a method for mancozeb analysis, which is why mancozeb is not measured.

5.2.7.1 Extraction

The rat plasma samples were extracted with acetonitril. Precisely 0.50 g of rat plasma was weighed directly into extraction tubes, 20.0 ml of acetonil was added and then extracted for 2 min. by an Ultra-Turrax mixer. Hereafter, 5.0 g anhydrous sodium sulphate was added and the extraction went on for another 2 min. The sample was filtered and evaporated using a gentle stream

[4] Hypnorm®: fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml
Dormicum®: midazolam 5 mg/ml.
The mixture for anasthaesia is made from mixing 1 part Hypnorm® with 1 part sterile water and adding this to a mixture of 1 part Dormicum®+1 part sterile water.

of nitrogen to nearly dryness, and diluted with acetonitril to 0.50 ml. The extract was further analysed by GC-MS, LC-MS and LC-MS-MS.

5.2.7.2 GC-MS-NCI analysis (chlorpyrifos, bromopropylate and alphacypermethrin)

Aliquots of 1µl were analysed by ion trap GC/MS with chemical ionisation. Methane was used as reagent gas. Chlorpyrifos was measured by the masses 313 and 315, bromopropylate by the masses 78.3-82 and alphacypermethrin by the masses 207, 209 and 343. The sample extracts from each analytical series were analysed together and quantified using bracketing calibration curves at five concentration levels at 0.87, 2.78, 8.7, 27.8 and 55.5 pg/µl for chlorpyrifos and 32.6, 104, 326, 1041 and 2083 pg/µl for bromopropylate and alphacypermethrin.

5.2.7.3 LC-MS analysis (TCP)

Aliquots of 10µl were analysed by LC/MS. The mass spectrometer was operated with electrospray in the negative ion mode (ESI-). Detection was performed in Single Ion Monitoring (SIM) mode. SIM conditions for TCP were mass 195.40. The sample extracts from each analytical series were analysed together and quantified using bracketing calibration curves at four concentration levels at 2.60, 8.68, 26.0 and 86.8 pg/µl.

5.2.7.4 LC-MS-MS analysis (carbendazim and chlorpyrifos-oxon)

Aliquots of 10µl were analysed by LC/MS/MS. The mass spectrometer was operated with electrospray in the positive ion mode (ESI+). Detection was performed in Multiple Reacting Monitoring (MRM) mode. MRM conditions for carbendazim were mass 191.76?159.57 and for chlorpyrifos-oxon 335.41?279.46. The sample extracts from each analytical series were analysed together and quantified using bracketing calibration curves at four-five concentration levels at 2.60, 8.68, 26.0 and 86.8 pg/µl carbendazim and 0.87, 2.60, 8.68, 26.0 and 86.8 pg/µl for chlorpyrifos-oxon.

The method was partly validated together with the analysis of the samples. The validation was performed at four different concentration levels as double determination and with four repetitions. Recoveries, relative repeatability's (RSD_r), relative reproducibility's (RSD_R) and Limit of Determination (LOD) are shown in Table 12.

As seen in Table 12 it was not possible to validate the method for chlorpyrifos-oxon. Prior to the validation, a preliminary recovery study was performed on water and rat plasma, in order to see whether the method gave acceptable recoveries of the spiked amount of chlorpyrifos-oxon. These results showed recoveries between 103-125%. Furthermore, recoveries performed in the first analytical series were between 83-117 (analytical series was carried out). These recoveries were carried out on the same rat plasma as used in the preliminary study. This rat plasma was provided from other control animals.

The rest of the recoveries were carried out on plasma from group 1 of the first subset of animals. These recoveries were very low and it seems like this plasma has degraded the added chlorpyrifos-oxon. It is likely that the content of the enzyme paraoxonase is responsible for degrading the added chlorpyrifos-oxon (see Table 12). The plasma used for the initial studies was quite old and maybe the paraoxonase was inactive. The plasma from group 1 has been stored at – 80 degree and therefore the paraoxonase has properly

been very active and has rapidly degraded (under 30 min.) chlorpyrifos-oxon. However further investigation should be carried out.

For the other compounds the RSD_r and RSD_R were acceptable and below the relative reproducibility derived from the Horwitz equation (12).

Table 12. Spike level (ng/g), recoveries (%), relative repeatability's (RSD_r, %), relative reproducibility's (RSD_R, %) and Limit of Determination (LOD, ng/g) for alphacypermethrin, bromopropylate, chlorpyrifos, chlorpyrifos-oxon, TCP and carbendazim.

Alphacypermethrin	Spike level, ng/g	72.9	208	729	2083
	Recovery, %	209	146	100	100
	RSD_r, %	21	4	3	5
	RSD_R, %	17	18	17	13
	LOD, ng/g	83			
Bromopropylate	Spike level, ng/g	72.9	208	729	2083
	Recovery, %	127	78	83	78
	RSD_r, %	10	17	9	21
	RSD_R, %	11	15	29	20
	LOD, ng/g	30			
Chlorpyrifos	Spike level, ng/g	0.97	2.8	9.7	2.78
	Recovery, %	147	119	155	141
	RSD_r, %	10	18	18	11
	RSD_R, %	27	32	31	26
	LOD, ng/g	1.1			
Chlorpyrifos-oxon	Spike level, ng/g	0.97	2.8	9.7	2.78
	Recovery, %	0	17	1	23
	RSD_r, %	113	80	128	142
	RSD_R, %	205	148	167	112
	LOD, ng/g				
TCP	Spike level, ng/g	0.97	2.8	9.7	2.78
	Recovery, %	97	97	119	95
	RSD_r, %	7	4	3	15
	RSD_R, %	36	24	9	12
	LOD, ng/g	1.0			
Carbendazim	Spike level, ng/g	1.1	3.1	10.9	31.0
	Recovery, %	80	74	78	76
	RSD_r, %	12	4	7	5
	RSD_R, %	36	26	11	15
	LOD, ng/g	0.9			

5.2.8 Terminal procedures

5.2.8.1 Necropsy and macroscopic pathology

All animals were subjected to necropsy. The order of necropsy was balanced with respect to dose group and sex. The animals were euthanized by decapitation in Hypnorm®/Dormicum® anaesthesia. A full macroscopic examination was performed and all lesions were recorded.

5.2.8.2 Organ weights

The following organs of all animals were weighed: liver, kidneys, adrenal glands, spleen, thymus, thyroid glands, testes, epididymis (males only), heart and brain. The last of the weekly body weight measurements, obtained on the Monday of the week of sacrifice, was used for calculation of relative organ weights.

5.2.8.3 Histology

The following tissues were formaldehyde preserved and evaluated by histopathology: intestinal tract, liver, kidneys, adrenal glands, spleen, thymus, thyroid glands, trachea, lungs, gonad's, accessory sexual organs, bladder, lymph nodes, heart, bone marrow, peripheral nerve, spinal cord, left half of the brain and in addition tissues with macroscopically lesions.

5.2.9 Data evaluation

Data from treated animals were compared with control data. Males and females were evaluated separately.

No statistical analysis was done on data where it was obvious that no group difference was present (some FOB data, eye examination data).

Continuous data were evaluated with respect to conformity with normal distribution and variance homogeneity, and it was determined whether a parametric or a non-parametric test would be more suitable. Parametric data were analysed by a parametric analysis of variance followed by Dunnetts test. Non-parametric data were analysed by Wilcoxons test of one-way analysis of variance followed by Kruskal Wallis test. Differences with $P < 0.05$ were regarded statistical significant.

5.3 Results

5.3.1 Clinical signs, morbidity and mortality

No adverse clinical signs were observed. Two animals died during the study (one male in group 1, one male in group 5) while anaesthetized for blood sampling for haematology during the 4th week of dosing. These deaths are regarded as unrelated to the exposure to test substances.

5.3.2 Body weights

All groups gained weight during the study period (statistically significant effect of time). There was no statistically significant difference among groups within each sex.

5.3.3 Relative food and water consumption

All groups showed the normal decrease in relative food and water consumption during the study period (statistically significant effect of time). There was no statistically significant difference among groups within each sex.

5.3.4 Functional observational battery (FOB)

No unusual behaviour was observed. The only statistically significant difference was an 11% reduced forelimb grip strength in males of group 5 compared with vehicle control. However, as no dose response was found, this is considered unrelated to treatment.

5.3.5 Motor activity measurement

The variability in the data was large, which is commonly observed. Total motor activity during 30 minutes was unaffected by treatment. In all groups, a

statistical significant decline in activity over time was found indicating that habituation to the novel situation occurred and this is a normal finding. There was no difference between treatment groups in the repeated measures analysis of individual 5-minute activity periods.

5.3.6 Eye examinations

No treatment-related abnormalities were observed. Persistent pupillary membrane was observed in 9 rats at the first assessment (May 15 and 16, before start of exposure). This is regarded as a normal finding. The distribution was: group 1: 3 males (nos. 1, 6, 17), group 2: 1 male (no. 34), group 3: 2 males (nos. 65, 70), group 4: 1 male (no. 101), group 5: 1 male (no. 129) and 1 female (no. 150), group 6: 1 male (no. 164). At the second assessment (June 12) during exposure week 4, where only groups 1 and 6 were assessed, no incidence of persistent pupillary membranes were observed, except in one male rat (no. 6 of group 1). In addition to the observations made during the scheduled assessments of group 1 and 6 during exposure week 4, a pathological finding was observed in one rat (no. 37, male, group 2) whose right eye was severely enlarged and opaque. This is regarded as an incidental finding and is probably caused either by spontaneously occurring glaucoma, which has been observed in rats of this strain before, or by trauma.

5.3.7 Acetylcholinesterase activity

5.3.7.1 Plasma acetylcholinesterase activity

A slight but statistically significant lower activity in plasma acetylcholinesterase was found in males of group 2, 5 and 6 compared with the vehicle control group (see Figure 2). The plasma acetylcholinesterase level was approximately three times higher in females compared to males (see Figure 2), this is a normal finding.

5.3.7.2 Relative brain acetylcholinesterase activity

No treatment-related difference was found. The relative brain acetylcholinesterase level was the same in females and in males.

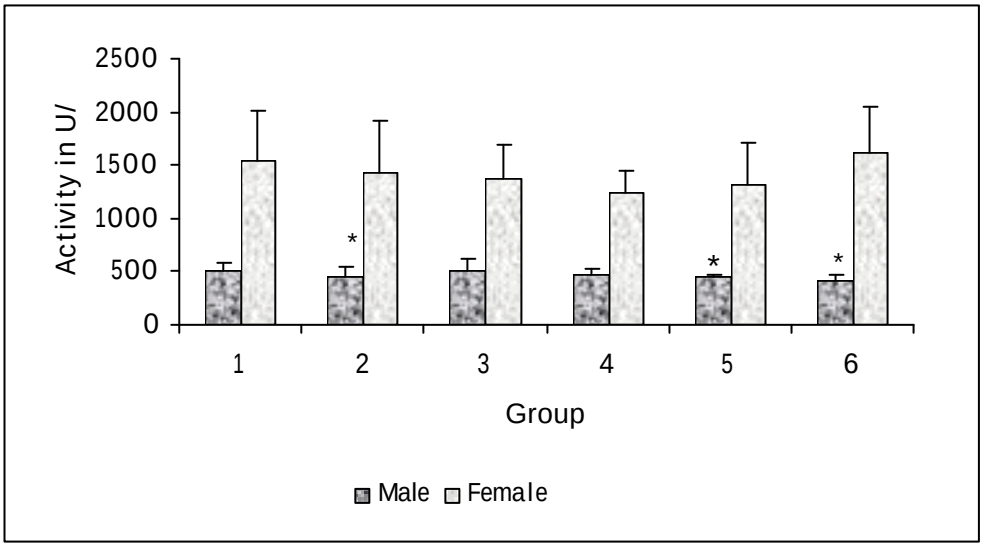

Figure 2. Plasma acetylcholinesterase activity (units/L) in male and female rats in the 6 dose groups. See dose group in table 8. * Statistical significant different (P < 0.05) from group 1 (control)

5.3.8 Plasma pesticides concentrations

Chlorpyrifos was found in some samples but in very low concentration below the determination limit (1 ng/g). The degradation product from chlorpyrifos, chlorpyrifos-oxon was not found in any samples, but TCP, another degradation product, was found in all samples from group 2-6 (see Figure 3). The concentrations seem to be dose dependent. The concentration found in samples from the satellite study seems to be lower than the concentration in the samples from the main study. No differences were observed between males and females.

No alphacypermethrin, bromopropylate and carbendazim were detected in group 1 or 2. Bromopropylate was found in all samples analyses from groups 3-6 with a mean concentration of 263 ng/g ranging between 54-1383 ng/g (see Figure 4). The concentration found in samples from the satellite study seems to be lower than the concentration in the samples from the main study. No differences were observed between males and females.

Alphacypermethrin was found in most of the samples (63 out of 68) from groups 3-6 (see Figure 5). The mean concentration was 247 ng/g with result between 0-636 ng/g. No differences were observed between the main and satellite study or between males and females.

Carbendazim was found in almost half of the samples (30 out of 68) from groups 3-6 (see Figure 6). The mean concentration was 0.8 ng/g with result between 0-8 ng/g. No differences were observed between the main and satellite study or between males and females.

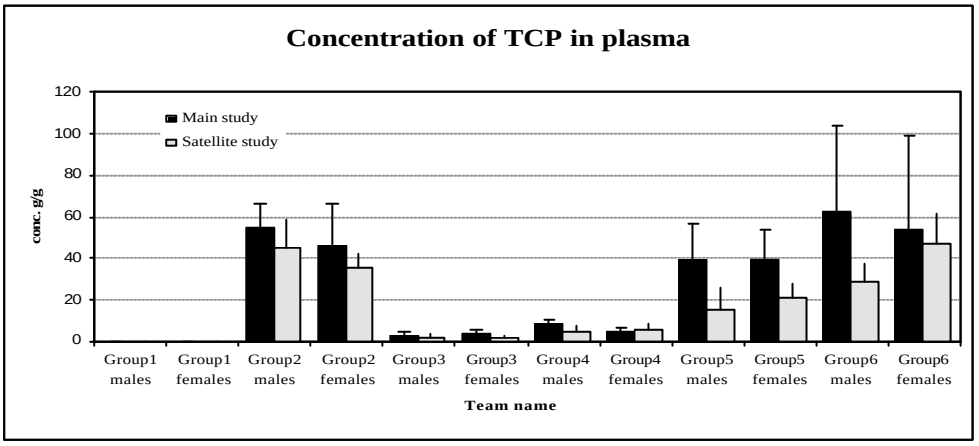

Figure 3. Plasma pesticide concentration of the metabolite TCP of chlorpyrifos after 1 week (satellite study) and after 4 weeks (main study) in male and female rats for the 6 dose groups. See dose groups in Table 8.

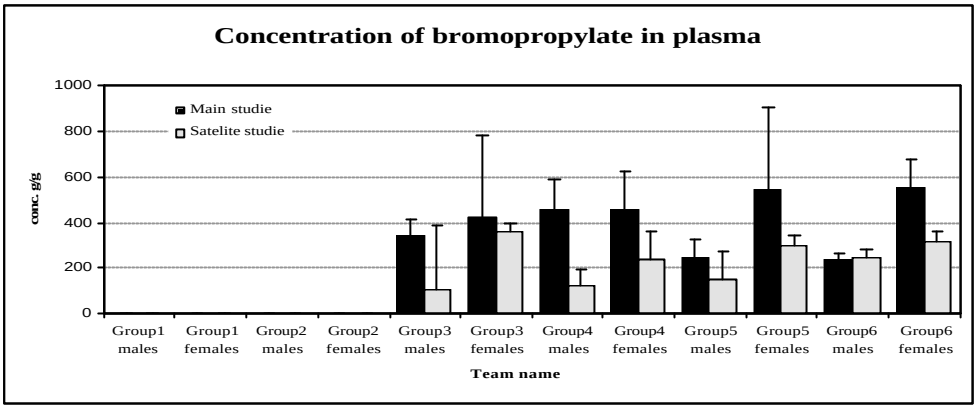

Figure 4. Plasma pesticide concentration of bromopropylate after 1 week (satellite study) and after 4 weeks (main study) in male and female rats for the 6 dose groups. See dose groups in Table 8.

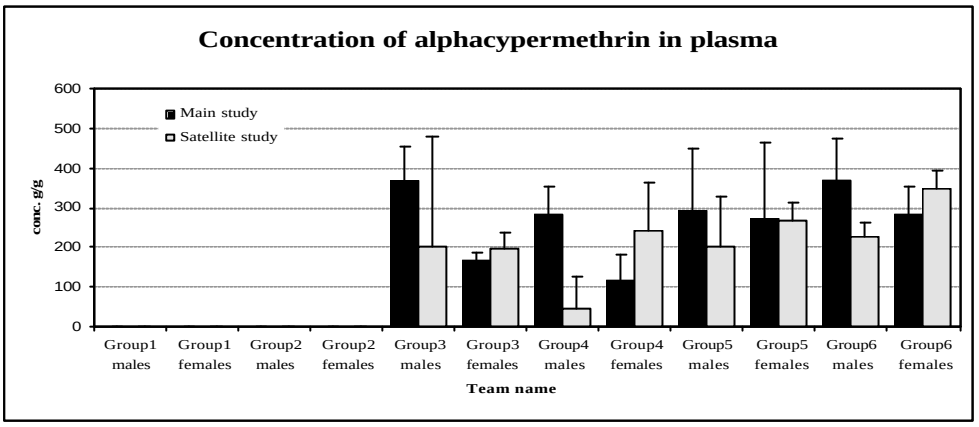

Figure 5. Plasma pesticide concentration of alphacypermethrin after 1 week (satellite study) and after 4 weeks (main study) in male and female rats for the 6 dose groups. See dose groups in Table 8.

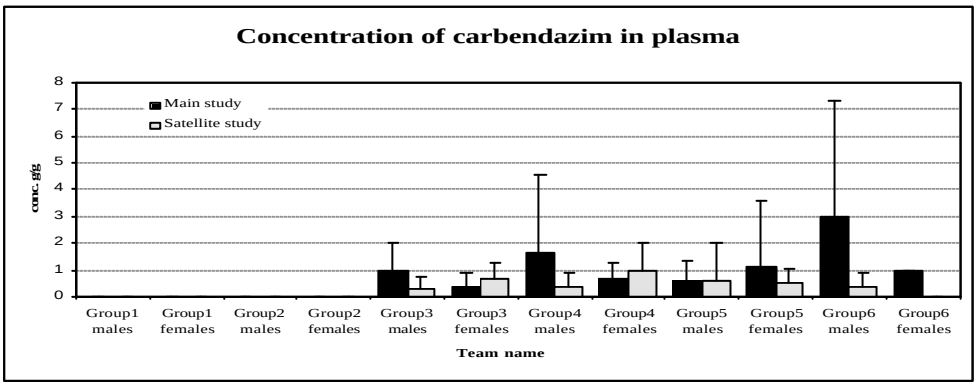

Figure 6. Plasma pesticide concentration of carbendazim after 1 week (satellite study) and after 4 weeks (main study) in male and female rats for the 6 dose groups. See dose groups in Table 8.

5.3.9 Haematology

There were no differences in white blood cell count (WBC) or differential count for male rats. Group 6 female rats had a significantly lower WBC, but there was no statistically significant difference in differential WBC counts (see Appendix C, Table 1-4C).

There was a significant reduction in red blood cell count (RBC) of group 3, 5 and 6 male rats and in group 5 and 6 female rats. There was a significant reduction in haemoglobin (Hb) in group 3, 4, 5 and 6 male rats and in group 2, 5 and 6 female rats. There was a significant reduction in haematocrit (Hct) in group 2, 3, 4, 5 and 6 male rats and group 2, 5 and 6 female rats. There were no significant changes in mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) in any of the groups except for group 2 female rats where there is a higher MCHC (see Appendix C, Table 1C and 3C).

5.3.10 Pathology

5.3.10.1 Necropsy and macroscopic pathology

There were two un-scheduled deaths, one male rat in group 1 and one male rat in group 5 died following blood sampling after Hypnorm-Dormicum sedation. At autopsy they were found to have enlarged haemorrhagic livers and large adrenal glands.

Macroscopic findings:

None of the macroscopic lesion was dose-related. In group 2 males, one male had bilateral increased size of lnn. Axillaris, this male also had glaucoma of the right eye. In group 2 females, one female was very thin (No. 52) and two females had a superficial skin wound in the inguinal area. In group 3 males, one male had a very small (atrophic) right adrenal gland. In group 5 females, one female had a superficial skin wound in the inguinal area. In group 6 males, one male had a superficial skin wound in the inguinal area and in group 6 females; one female had a superficial skin wound in the inguinal area. All of the above mentioned superficial skin wounds were caused by the injection with Hypnorm-Dormicum.

5.3.10.2 Organ weights

Body weight and absolute and relative organ weights for brain, heart, liver, spleen, kidneys, adrenal glands, thymus, thyroid gland, uterus, ovaries, testis and epididymis are presented in Appendix C, Table 5-8C as group means with standard deviation. All organ weights except the weight of the thyroid gland were normally distributed.

There was a statistical significant increase in relative liver weight in group 3-6 in both male and female rats. There was a statistically significant increase absolute weight of the Thyroid gland in group 3, 5 and 6 in male rats and in group 3-6 in female rats. There was a statistically significant increase in relative weight of the thyroid gland in group 2-6 in male rats and group 3-6 in female rats. There was a statistically significant decrease in absolute weight of the thymus in group 3-6 in both male and female rats, and a decrease in relative thymus weight in group 3-6 in male rats and group 3 and 4 in female rats.

5.3.10.3 Histopathology

All organs were evaluated by histopathology in the controls (group 1) and in the highest dose group (group 6). All the histopathological findings and remarks are shown in Appendix C under histopathology Table 9C. In the liver, 8 out of 8 males in group 6 had a mild degree of centrilobular cell hypertrophy and 3 out of 8 females in group 6 had a mild degree of centrilobular cell hypertrophy. None of the livers of group 1 males and females showed these changes. One to three Foci of inflammatory cells were found in 7 out of 7 males in group 1, 6 out of 8 males in group 6, 4 out of 8 females in group 1 and 5 out of 8 females in group 6. In the thyroid gland 1 out of 8 females in group 1, 6 out of 8 females in group 6 and 7 out of 8 males in group 6 had a mild degree of follicular cell hypertrophy.

An increased number of animals in the high dose group (group 6) have dose-related findings with a mild degree of centrilobular cell hypertrophy in the liver and a mild degree of follicular cell hypertrophy in the thyroid gland. The histological changes in the liver could indicate an increased cytochrome-P450 activity. The histological changes in the thyroid gland indicate an increased activity probably stimulated by increased levels of TSH. The weight changes in the thymus did not reflect any histological changes. The decrease in weight of thymus might be the beginning of an involution of the thymus not yet recognizable histological. The histological finding of foci of inflammatory cells in the liver in the combined exposure study is not considered substance related as this finding was seen in both control and high dose group.

5.4 Conclusions of the combined exposure study

It was hypothesised that the effect of chlorpyrifos on brain acetylcholinesterase inhibition is enhanced by the co-administration of the other four pesticides: alphacypermethrin, bromopropylate, carbendazim and mancozeb. The primary objective of this study was to confirm or reject the hypothesis and if possible show a dose-response of brain acetylcholinesterase inhibition by varying the dose of chlorpyrifos and keeping the four other pesticides at constant concentration. The second objective was to examine the possibility of increased liver metabolism of chlorpyrifos to the active metabolite chlorpyrifos oxon.

It is concluded that it was not possible to confirm the hypothesis that the effect of chlorpyrifos on brain acetylcholinesterase inhibition is enhanced by co-administration of the other four pesticides. It was therefore not possible to show a dose-response of chlorpyrifos in combination with the four other pesticides. However, some combination effects were observed, since there were increased weights of liver and the thyroid gland including histological changes in these organs. There was a decreased weight of the Thymus without histological changes. Furthermore some changes in haematology were found. These findings will be discussed below.

It cannot be concluded that the mode of action is through increased liver metabolism of chlorpyrifos to the active metabolite chlorpyrifos-oxon. Chlorpyrifos-oxon could not be found in plasma and TCP increased in a dose-related manner. The increase in relative liver weight together with a mild degree of centrilobular cell hypertrophy is indicative of liver enzyme induction. However, the mode of action might be through other mechanisms than chlorpyrifos metabolisation in the liver.

6 Discussion

6.1 CNS-symptoms and brain acetylcholinesterase

No dose related changes in clinical appearance were observed in either the dose-response study of chlorpyrifos or the combined exposure study of the five pesticides. Thus, the clinical observation of severe toxic effect on the central nervous system seen in the initial study was not confirmed. The observation that the rats at autopsy in the initial study appeared more sensitive to sound stimuli after 28 days exposure of the mixture of the five pesticides at the NOAEL-level was not seen in the combined exposure study even though group 5 in the combined exposure study represented a similar dose level as the mid-dose group in the initial study.

Two of the pesticides in the mixture are known to affect CNS. Chlorpyrifos acts by inhibiting acetylcholinesterase and signs of intoxication are stimulation of the parasympatic nerve system. Alphacypermethrin acts by changing the sodium channels by increasing the permeability in the nerve membranes, which causes a general stimulation of neurotransmitters. Since the brain acetylcholinesterase was reduced by 8% and 12% in the low and mid-dose group in the initial study, chlorpyrifos was suspected to be the cause of the intoxication. However, the initial study was performed in 1998 and in 2000 the JMPR evaluation considered only statistically significant inhibition of ± 20% of brain acetylcholinesterase to represent a clear toxicological effect (39). Preliminary results, from an ongoing experiment in our laboratory, shows that chlorpyrifos dosed subcutaneous once a week in doses up to 30 mg/kg induced inhibition of brain acetylcholinesterase of approximately 60% without any CNS symptoms. Therefore it can be questioned whether an inhibition of brain acetylcholinesterase of 8% and 12 % in the initial study was the cause of the CNS-symptoms seen. It cannot be excluded that alphacypermethrin contributed to the observed effects on CNS in the initial study, since it is known that alphacypermethrin cause increased sensitivity to sounds and causes high stepping and splayed gait (37). However, in the combined exposure study the data from the behavioural tests and the measurement of motor activity did not indicate any substance related change in the function of the nervous system. Another explanation for the lack of CNS-symptoms and inhibition of acetylcholinesterase in the combined exposure study could be the low dose of mancozeb in the diet only between 18-26 % of the intended. A combination effect of mancozeb and chlorpyrifos and/or alphacypermethrin can therefore not be excluded. Finally the lack of CNS symptoms in the combined exposure study could be a consequence of lowering the high dose from five times the NOAEL of all the pesticides in the initial study to two times NOAEL of chlorpyrifos and the level of NOAEL of the other 4 pesticides in the mixture in the combined exposure study.

6.2 Plasma acetylcholinesterase

The data in the combined exposure study did reveal a slight statistically significant reduction in plasma acetylcholinesterase activity in the males of group 2, 5 and 6. This is considered to be without biological significance due

to the very low levels. Preliminary results from an ongoing experiment at Institute of Food Safety and Nutrition show that a reduction of approximately 45% in plasma acetylcholinesterase activity is without change in clinical behaviour.

6.3 Plasma pesticide concentration

Plasma pesticide was measured in blood of alphacypermethrin, bromopropylate, carbendazim and chlorpyrifos after one week and after four weeks. The plasma pesticide concentrations found in samples from the first subset of animals euthanized after one week in the combined exposure study seems to be lower than the concentration in the blood samples taken after four weeks. This difference probably reflects the change in steady state levels from one week of exposure to four weeks of exposure. Mancozeb was not measured in plasma. It was planned to develop and validate a method for measuring mancozeb. However, plasma needed to be stabilized in EDTA and unfortunately we only obtained enough blood from few animals to stabilize blood both in heparin and EDTA.

The active metabolite of chlorpyrifos, chlorpyrifos-oxon, was not found in any samples, but the metabolite TCP was found in all samples from group 2-6 in the combined exposure study. The concentrations seemed to be dose dependent. There did not seem to be a combination effect since the levels were similar in group 2 (NOAEL of Chlorpyrifos) and group 5 (NOAEL of chlorpyrifos, bromopropylate, alphacypermethrin, carbendazim, mancozeb). Some reservations have to be taken for the data on chlorpyrifos-oxon, as there apparently were problems with the methods. It seemed like the use of plasma from the group 1 animals in the first subset caused a degradation of the oxon compounds. This could be due to the enzyme paraoxonase, which rapidly degrade chlorpyrifos-oxon (17). However, further investigation is needed.

6.4 Diet pesticide concentration

Pesticide concentrations were measured in the diet of the combined exposure study. Alphacypermethrin, bromopropylate and carbendazim were found at acceptable levels of the intended (60-90%). Chlorpyrifos was found at acceptable levels in group 4-6 (80-135%), but the concentration in group 3 was 300% higher than intended. Mancozeb was only found at levels of 18-26% of the intended. The result from the analysis of the diet was very disappointing, because the low level of mancozeb makes comparison between the initial study and the combined exposure study very difficult. Analysing the diet retrospectively for the intended content of pesticides as done in the combined exposure study is truly a problem when the content deviate op to 300% from the intended. The stability of the pesticides in the diet is another problem some degradation of mancozeb cannot be excluded, as the diet was stored at a room temperature of 16-18°C.

The reason for dosing the pesticides through the diet was to resample the human situation, where pesticide residues are consumed in the diet through fruit and vegetables. There are advantages and disadvantages by dosing the animals through the diet. One of the advantages of feeding through the diet is that the intake of the compound is continuously dosed to the animals, whereas dosage by gavage is given as a bolus once or twice a day. Disadvantages is that it can only be estimated how much the rat eats of the diet. Two rats were housed together in a cage due to animal welfare. However, one of the rats in a

cage is the dominating animal and will eat more than the other rat. When dosing by gavage it is known exactly what the rat gets which is an advantage.

6.5 Organ weight changes

6.5.1 Liver

The relative organ weight of the liver was increased in both sexes in the combination groups (group 3-6) in the combined exposure study. This indicates that the four pesticides rather than chlorpyrifos are responsible for the weight increase. Others have found increase in liver weight at high dose levels for bromopropylate, carbendazim and mancozeb (35;36;38).

Histological examination revealed a mild degree of centrilobular cell hypertrophy in 8 out of 8 males and 3 out of 8 females in group 6 in the combined exposure study. This is a normal histological finding when liver microsomal enzymes like cytochrome-P450 are induced (1;22). The relative liver weight increase in group 6 was between 17 and 20% in females and between 15 to 20% in males. This is in agreement with Amacher et al. (1998), who found that a weight increase of 20 % and above was associated with hypertrophy of the liver. They found a correlation between increased liver weights and microsomal cytocrome-P450 enzyme induction (1). Both bromopropylate and carbendazim are known to induce liver enzymes (35;36). However, despite the indication of liver enzyme induction there was not an increase of the active metabolite chlorpyrifos-oxon in plasma. An increase in liver metabolism of chlorpyrifos might not be the mode of action of the toxicity seen in the initial study. However, liver metabolism cannot be excluded since there apparently were problems with the methods used to measure chlorpyrifos-oxon concentrations in the plasma.

Chlorpyrifos can be both activated by cytochrome P450 enzymes through a desulfuration reaction to form chlorpyrifos-oxon and detoxified through a dearylation reaction (31). Tang et al. found CYP2B6 to have the highest activation (desulfuration) activity, whereas CYP2C19 had the highest detoxification (dearylation) activity and CYP3A4 had a high activity for both activation and detoxification in human liver microsomes (31). One research group suggested CYP1A1 and CYP2B6 as possible metabolic biomarkers for organaphosphorothioate toxicity in humans (3), whereas another group found CYP2D6 to be important in the microsomal bio activation pathway of chlorpyrifos (26). Vodela et al. (1995) found chlorpyrifos to be an inhibitor of hepatic microsomal drug-metabolising enzymes in rats (33). There might be differences between human and rat metabolism of chlorpyrifos. Most of the above mentioned studies were performed in *in vitro* human liver microsomes. No cytochrome P450 enzymes were measure in the combined exposure study due to the lack of knowledge of which of the enzymes are involved in chlorpyrifos metabolism in rats. Further investigation on which of the cytochrome P450 enzymes that are important in activation and detoxification of chlorpyrifos in the rat liver microsomes is needed.

Differences between male and female human liver microsomes are seen with females possessing greater activity than males (31), this correspond the higher effect seen *in vivo* in our laboratory in female rats on brain acetylcholinesterase inhibition.

6.5.2 Thyroid

The absolute and relative organ weight of the thyroid gland in the combined exposure study was statistically significantly increased in groups 3-6 in females, absolute weight was significantly increased in group 3, 5 and 6 in males and the relative weight was increased in groups 2-6 in males. The histological examination of the thyroid gland revealed a mild degree of follicular cell hypertrophy in most animals in group 6 of both sexes. This indicates an increased activity in the thyroid gland in the combination group.

Of 240 pesticides screened by the U.S. Environmental Protection Office (EPA) of Pesticide Programs; 15% (37/240) induced effect on the thyroid follicular cells in rodents (13). It is well established that thyroid-stimulating hormone TSH is the main growth factor for thyroid cells maintaining the differentiated state of the thyroid and controlling thyroid hormone secretion (10). The control of the thyroid hormone in the blood is regulated by a negative feedback mechanism involving the hypothalamus, the pituitary and the thyroid gland. The hypothalamus releases thyrotropin-releasing hormone (TRH), which stimulates the pituitary to produce TSH. TSH stimulates the thyroid to produce thyroid hormones. Cells of both the hypothalamus and pituitary respond to levels of circulation thyroid hormone. When levels of thyroid hormones are low, the outputs of TRH and TSH are raised and the thyroid stimulated to increase the output of thyroxin (T_4) and triiodothyronine (T_3). This feedback loop helps to maintain thyroid hormone homeostasis (13). The thyroid gland is capable of meeting physiological demands for T_4 and T_3 up to a point. However, beyond that point continuous stimulation of the thyroid may lead to hyperplasia and hypertrophy, which eventually can lead to neoplasia (13). Mancozeb and to some extent bromopropylate are the two pesticides in the mixture known to effect the thyroid gland. It is remarkable that the weight increase of the thyroid gland and the histological changes were present in the combined exposure study despite the low content (18-26%) of mancozeb in the diet. Szepvolgyi et al. (1989) found a dose-dependant hyperplasia of the thyroid gland in rats exposed to mancozeb at 113 mg/kg bw/day and above (30). In combined exposure study the weight increase and the histological changes in the thyroid gland indicate that the toxicity of mancozeb may be enhanced by the other four pesticides.

There are many ways by which chemicals can perturb the thyroid-pituitary homeostasis. Inside the thyroid these are: 1) inhibition of the active transport of inorganic iodide into the follicular cells; 2) inhibition of thyroid peroxidase (TPO) that converts inorganic iodide into organic iodide and couples iodinated tyrosyl moieties into thyroid hormone; 3) damage to follicular cells; and 4) inhibition of thyroid hormone release into the blood (13). Outside the thyroid gland these are: a) inhibition of conversion of T_4 to T_3 by 5'-monodeiodinase at various sites in the body and b) enhancement of the metabolism and excretion of thyroid hormones by the liver (13;23). Mancozeb has both intrathyroidal and extrathyroidal sites of action. According to the U.S. EPA Office of Pesticide program, mancozeb caused thyroid follicular cell hypertrophy and hyperplasia, increased the thyroid weight, decreased hormone levels of T_4 and T_3 and increased levels of TSH, decreased iodide uptake and thyroid peroxidase activity (13).

The changes in weight and histology of the thyroid gland in the combined exposure study indicate that the toxicity of mancozeb is enhanced. This could be caused partly by increased metabolism of mancozeb to the metabolite ETU

or increased metabolism of the thyroid hormones in the liver, which could perturb the thyroid homeostasis by increasing TSH-secretion. Male rats are more sensitive than female rats with regard to effects on thyroid hormones (13), as it was the case in the combined exposure study.

6.5.3 Thymus

Both absolute and relative weight of the thymus was reduced in all combination groups (group 3-6) in the combined exposure study. There was no weight change in the control group and in the chlorpyrifos group (group 2). In addition, the low organ weights were comparable between groups 3 to 6. This could indicate that the four pesticides (alphacypermethrin, bromopropylate, carbendazim and mancozeb) are responsible for this effect in combination at their respective NOAEL levels. There were no recognisable histological changes. The weight reduction of the thymus could be indicative of the beginning of an involution of the thymus which could be stress related (9;19) or caused by toxicity of one or more of the pesticides. The pesticide deltamethrin is, as alphacypermethrin, another member of the pyrethroid family and is known to cause thymus atrophy by apoptosis (6), and cypermethrin can induce alteration in thymocyte distribution in prenatal exposed rats (27). However, mancozeb is to our knowledge the only pesticide in the combination that is known to directly have affected thymus in dogs at dose levels at 1000 ppm or above. In that study a decrease in thymus weight was observed and the histological examination revealed a thymic cortical depletion (35).

6.6 Blood

The haematological data showing reduction in haematocrite (Htc), haemoglobin (Hb) and red blood cells (RBC) indicate that the exposed male animals in groups 3, 5 and 6 of the combined exposure study suffer from a mild degree of anaemia (4-7%). Since no dose related changes were observed in MCV, MCH and MCHC this anaemia is characterised as normocytic. The initial study did not reveal a similar effect, and there is no obvious reason for this discrepancy. However, the mild degree of the anaemia together with the lower number of animals per group in the initial study could partly account for the different result. Both alphacypermethrin, carbendazim and chlorpyrifos are known to cause a reduction in the number of erythrocytes in rats (36-38). In the beagle dog, mancozeb have caused anaemia in two different studies at dose levels of 113 mg/kg bw/day (35). Anaemia may result from a reduced rate of production, increased rate of destruction of red cells or loss of red blood cells from the circulation due to bleeding (34). The blood has a large regenerative capacity and whether a degree of anaemia of 4-7% is an adverse effect can be discussed. However, the mild anaemia observed in male rats in group 3, 5 and 6 in the combined exposure study supports the theory of a combination effect.

In the initial study the number of white blood cells (WBC) was reduced significantly in males in the mid-dose group. In the combined exposure study a significant reduction in WBC was seen in females in the high dose group. In the initial study the number of neutrophils was increased in males in the mid-dose group, but in the combined exposure study there was not a shift in the differential count. Because the changes in WBC were seen in different sexes in the two studies and there is inconsistency in change of differential counts, this effect could be non-specific or considered incidental. However,

carbendazim is known to decrease WBC at high dose levels (38), and a combination effect cannot be excluded.

6.7 Risk assessment

In general the combined exposure study showed statistically significant effects on organ weights for the thymus, the thyroid gland and the liver and histological changes in the liver and the thyroid gland, as well a haematological changes. These changes were found at levels of NOAEL of the individual compounds. At NOAEL of the individual compounds none of these changes were present in the literature (35-38). Thus the effects in the combined exposure study could be interpreted as a combined effect. Effects are seen at lower levels when rats are exposed to the five pesticides in combination than when the rats are exposed to the individual pesticides. On basis of the two present combination studies a true NOAEL is not found, which makes risk assessment difficult. Using NOAEL of the individual pesticides it is important with a large safety factor on ADI. This gives a greater safety margin when risk assessment for adverse health effects is evaluated. The suggested method for evaluation of combined effects by assumption of an additive effect (14) and the method used by The Danish Veterinary and Food Administration (25) seems to give some certainty that there is no health hazard by eating food items containing small amounts of more than one pesticide.

7 Overall conclusions

Effects are seen at lower levels when rats are exposed to the five pesticides in combination than when the rats are exposed to the individually pesticide. This complicates risk assessment for exposure of a combination of pesticides. The assumption of an additive effect as used as a basis for health assessment of residues of pesticides in food seems appropriate and feasible.

The unespected effect on CNS and on brain acetylcholinesterase seen in the initial study could not be repeated in the combined exposure study. However, since the level of mancozeb in the diet in the combined exposure study was only 18-26% of the intended, the effect on CNS in the initial study still might be a true effect.

Combination effects were seen in the combined exposure study, as there was weight increase in the relative liver weight, the thyroid gland weight and a decrease in the weight of the thymus in the combination groups. There were changes in the histology of the liver and the thyroid gland and changes in both white and red blood cells in the combination groups.

Increased liver metabolism of chlorpyrifos to the active metabolite chlorpyrifos could not be confirmed, since the metabolite chlorpyrifos-oxon could not be found in plasma. However, there were some problems with the method used to measure chlorpyrifos. The effects seen in the initial study might be trough other mechanisms e.g. change of toxico-kinetics.

8 Perspectives

From a regulatory point of view the CNS symptoms seen in the initial study still gives reason to concern, and there is a need to explain the findings. Which of the pesticides in the combination gave the CNS symptoms alphacypermethrin, chlorpyrifos or a combination? Since the level of mancozeb in the diet in combined exposure study was only 18-26% of the intended it cannot be excluded that the toxicity was caused by mancozeb in combination with chlorpyrifos and/or alphacypermethrin.

There is furthermore a need to perform some toxicokinetic studies of the single compounds and compounds in combination. Toxicokinetic studies might elucidate possible modes of action, since there might be changes in absorption, distribution, metabolism and elimination of a single compound in the combinations.

To support the toxicokinetic studies, further investigation on which of the cytochrome P450 enzymes that are important in activation and detoxification of chlorpyrifos or the other pesticides in the rat is needed.

This emphasizes the complexity of combination studies. In the ideal combination toxicity study there are dose-response and toxicokinetic studies of each compound and of all possible combinations of the compounds. This might make it possible to predict which of the compounds are responsible for the changes seen in the combination. However, from an ethical point of view this is unacceptable, since it would require a large number of animal experiments.

9 List of used abbreviations

AChE: Acetylcholinesterase
ADI: Acceptable daily intake
AL: Alfacypermetrin
ALAT: Alanine aminotransferase
ALP: Alkaline phosphatase
B: Polymorphonuclear basophils
BR: Bromopropylat
CB: Carbendazim
CH: Chlorpyrifos
GC-MS: Gas Chromatography with Mass Selective Detection
GC-MS-NCI: Gas Chromatography with Mass Selective Detection with Negative Chemical Ionisation
E: Polymorphonuclear eosinophils
ESI-: Electro Spray in the Negative ion mode
ESI+: Electro Spray in the Positive Ion Mode
ETU: Ethylene thiourea
FOB: Functional observational battery
GFAP: Glial fibrillary acidic protein
Hb: Haemoglobin
Hct: Haematocrit
HE: Haematoxylin and eosin
JMPR: Joint Meeting on Pesticide Residues
L: Lymphocytes
LC-MS: Liquid Chromatography with Mass Selective Detection
LC-MS-MS: Liquid Chromatography with Tandem Mass Selective Detection
LOD: Limit of Determination
M: Monocytes
MA: Mancozeb
MCH: Mean corpuscular haemoglobin
MCHC: Mean corpuscular haemoglobin concentration
MCV: Mean corpuscular volume
MRM: Multiple Reacting Monitoring
MS: Mass spectrometry
N: Polymorphonuclear neutrophils
NOAEL: No-Observed-Adverse-Effect-Level
NOEL: No-Effect-Level
RBC: Red blood cell count PLT: Platelet count
RSD_r: Relative repeatability. By repeatability means precision under repeatability conditions. Repeatability conditions means conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment on the same day.
RSD_R: Relative reproducebility. By reproducibility means precision under reproducibility conditions. Reproducibility conditions means conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment on different days.
SIM: Single Ion Monitoring

TCP: 3, 5, 6-trichloro-2-pyridinol
TSH: Tyroid-Stimulating Hormone
TRH: Thyrotropin-Releasing Hormone
T_4: Thyroxine
T_3: Triiodthyronine
WBC: White blood cell count
WHO: Whorld Health Organisation

10 References

(1) Amacher DE, Schomaker SJ, Burkhardt JE. The relationship among microsomal enzyme induction, liver weight and histological change in rat toxicology studies. Food Chem Toxicol 1998; 36(9-10):831-839.

(2) Belpoggi F, Soffritti M, Guarino M, Lambertini L, Cevolani D , Maltoni C. Results of long-term experimental studies on the carcinogenicity of ethylene-bis-dithiocarbamate (Mancozeb) in rats . Ann N Y Acad Sci 2002; 982:123-136.

(3) Buratti FM, Volpe MT, Meneguz A, Vittozzi L, Testai E. CYP-specific bioactivation of four organophosphorothioate pesticides by human liver microsomes. Toxicol Appl Pharmacol 2003; 186(3):143-154.

(4) Ellman GL, Courtney KD, AV, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961; 7:88-95.

(5) Emea The European Agency for the Evaluation of Medicinal Products. Committee for veterinary medicinal products-alphacypermethrin summary report (2). 2. 2001.

(6) Enan E, Pinkerton KE, Peake J, Matsumura F. Deltamethrin-induced thymus atrophy in male Balb/c mice. Biochem Pharmacol 1996; 51(4):447-454.

(7) FAO. Pesticide residues in food- Evaluations 1981. FAO Plant Production and Protection Paper 42, 79-111. 1981.

(8) Fødevaredirektoratet. Pesticidrester i fødevarer 2001. FødevareRapport 2002:25. 2002.

(9) Gorski JR, Muzi G, Weber LW, Pereira DW, Iatropoulos MJ, Rozman K. Elevated plasma corticosterone levels and histopathology of the adrenals and thymuses in 2,3,7,8-tetrachlorodibenzo-p-dioxin-treated rats. Toxicology 1988; 53(1):19-32.

(10) Hard GC. Recent developments in the investigation of thyroid regulation and thyroid carcinogenesis. Environ Health Perspect 1998; 106(8):427-436.

(11) Henschler D, Bolt HM, Jonker D, Pieters MN, Groten JP. Experimental designs and risk assessment in combination toxicology: panel discussion. Food Chem Toxicol 1996; 34(11-12):1183-1185.

(12) Horwitz W. Evaluation of analytical methods used for regulation of foods and drugs. Anal Chem 1982; **54**,(1):67A-76A.

(13) Hurley PM. Mode of carcinogenic action of pesticides inducing thyroid follicular cell tumors in rodents. Environ Health Perspect 1998; 106(8):437-445.

(14) Ikeda M. Multiple exposure to chemicals. Regul Toxicol Pharmacol 1988; 8(4):414-421.

(15) JECFA. Toxicological evaluation of certain veterinary drug residues in food. WHO Food Additives Series 38, 133-170. 1996. WHO Food additives series 38.

(16) Konemann WH, Pieters MN. Confusion of concepts in mixture toxicology. Food Chem Toxicol 1996; 34(11-12):1025-1031.

(17) Li WF, Furlong CE, Costa LG. Paraoxonase protects against chlorpyrifos toxicity in mice. Toxicol Lett 1995; 76(3):219-226.

(18) Ma T, Chambers JE. Kinetic parameters of desulfuration and dearylation of parathion and chlorpyrifos by rat liver microsomes. Food Chem Toxicol 1994; 32(8):763-767.

(19) Morishita S, Sato EF, Takahashi K, Manabe M, Inoue M. Insulin-induced hypoglycemia elicits thymocyte apoptosis in the rat . Diabetes Res Clin Pract 1998; 40(1):1-7.

(20) Mumtaz MM. Risk assessment of chemical mixtures from a public health perspective. Toxicol Lett 1995; 82-83:527-532.

(21) Mumtaz MM, Sipes IG, Clewell HJ, Yang RS. Risk assessment of chemical mixtures: biologic and toxicologic issues. Fundam Appl Toxicol 1993; 21(3):258-269.

(22) Niho N, Shibutani M, Tamura T, Toyoda K, Uneyama C, Takahashi N et al. Subchronic toxicity study of gallic acid by oral administration in F344 rats. Food Chem Toxicol 2001; 39(11):1063-1070.

(23) Nordic Council of Ministers. The influence of chemicals in the food and the environment on the thyroid gland function. TemaNord 2002:520.

(24) OECD. Guidance Notes for Analysis and Evaluation of Repeat-Dose Toxicity Studies. Testing and Assement No. 32. Series on Pesticides No. 10. 2000.

(25) Reffstrup T.K. Combined actions of pesticides in food. FødevareRapport 2002; 2002:19.

(26) Sams C, Mason HJ, Rawbone R. Evidence for the activation of organophosphate pesticides by cytochromes P450 3A4 and 2D6 in human liver microsomes. Toxicol Lett 2000; 116(3):217-221.

(27) Santoni G, Cantalamessa F, Cavagna R, Romagnoli S, Spreghini E, Piccoli M. Cypermethrin-induced alteration of thymocyte distribution and functions in prenatally-exposed rats. Toxicology 1998; 125(1):67-78.

(28) Steenland K, Cedillo L, Tucker J, Hines C, Sorensen K, Deddens J et al. Thyroid hormones and cytogenetic outcomes in backpack sprayers using ethylenebis(dithiocarbamate) (EBDC) fungicides in Mexico. Environ Health Perspect 1997; 105(10):1126-1130.

(29) Sultatos LG, Murphy SD. Kinetic analyses of the microsomal biotransformation of the phosphorothioate insecticides chlorpyrifos and parathion. Fundam Appl Toxicol 1983; 3(1):16-21.

(30) Szepvolgyi J, Nagy K, Sajgone VK, Regoly-Merei A, Soos K, Toth K et al. Subacute toxicological examination of Dithane M-45. Food Chem Toxicol 1989; 27(8):531-538.

(31) Tang J, Cao Y, Rose RL, Brimfield AA, Dai D, Goldstein JA et al. Metabolism of chlorpyrifos by human cytochrome P450 isoforms and human, mouse, and rat liver microsomes. Drug Metab Dispos 2001; 29(9):1201-1204.

(32) The British Crop Protection Council. Cypermethrin (196). The e-Pesticide Manual (twelfth Edition) Version 2.0 12. 2000. Internet Communication.

(33) Vodela JK, Dalvi RR. Comparative toxicological studies of chlorpyrifos in rats and chickens. Vet Hum Toxicol 1995; 37(1):1-3.

(34) Weatherall DJ, Provan AB. Red cells I: inherited anaemias. Lancet 2000; 355(9210):1169-1175.

(35) WHO. Pesticide recidues in food - 1993. Joint FAO/WHO Meeting on Pesticide Residues in Food 1993; Part II - Toxicology.

(36) WHO. Pesticide recidues in food - 1995. Joint FAO/WHO Meeting on Pesticide Residues in Food 1995; Part II - Toxicological and Environmental.

(37) WHO. Evaluation of certain veterinary drug residues in food. Forty-seventh report of the joint FAO/WHO expert committee on food additives 1998; 876.

(38) WHO. Pesticide residues in food - 1999. Joint FAO/ WHO Meeting on Pesticide Residues 1999; Report 153.

(39) WHO. Joint Pesticide residues in food - 2000. Joint FAO/ WHO Meeting on Pesticide Residues 2000, Report.

Appendix A

Summary of the GLP report from the initial study (in danish).

The original GLP report with individual animal data can be obtained from :

The Danish Veterinary and Food Administration
Institute of Food safety and Nutrition
Moerkhoej Bygade 19, 2860 Soeborg, Denmark

Study report 994003
Study director: Otto Meyer

Instituttet for Fødevaresikkerhed og Toksikologi
Afdelingen for Almen Toksikologi

Rapport

Forforsøg til undersøgelse af mulig samspilseffekt ved samtidig eksponering af rotter for 5 pesticider, der kan optræde som rester i levnedsmidler

Sponsor

Veterinær- og Fødevaredirektoratet, Institut for Fødevaresikkerhed og Toksikologi, Mørkhøj Bygade 19.

Teststoffer

alfacypermethrin (CAS nummer 67375-30-8), bromopropylat (Novartis: GS19851A, technical, P.408159, 92.50%), carbendazim (AgrEvo code: technical, Hoe 017411 00 ZD99 0028), chlorpyrifos (CAS nummer 002921-88-2) & mancozeb (CAS nummer 8018-01-7).

Forsøget afsluttet

23. marts 1999

Rapport udarbejdet af

Otto Meyer, DVM

Laboratorium

Institut for Fødevaresikkerhed og Toksikologi

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1 Resumé

Rotter (MOL/Wist (Hannover), SPF), 3 hold af 6 hanner og 6 hunner per hold blev eksponeret for en kombination af pesticider via foder i overensstemmelse med OECD TG 407 med visse afvigelser. Udvidet observation, som foreskrevet i OECD TG 407, med henblik på undersøgelser for ændret adfærd blev ikke foretaget udover udvidet observation af dyrene uden for burene i forbindelse med vejning af dyrene og ved aflivningen.
Analyse for natrium og kalium i plasma blev ikke foretaget. I stedet for blev der analyseret for kalcium og cholinesterase aktivitet i plasma og cholinesterase aktivitet og protein i hjernehomogenat.
Chlorpyrifos (CH), bromopropylat (BR), alfacypermethrin (AL), carbendazim (CB) og mancozeb (MA) blev samlet iblandet pulveriseret chowfoder (Altromin), der blev slået til pellets, i koncentrationer, der for de enkelte stoffer svarede til 1/5 af NOAEL,
NOAEL og 5 gange NOAEL. Kontrolholdet på 6 hanner og 6 hunner fik samme foder dog kun iblandet vehikel (acetone).

Efter 1 døgns dosering udviste dyrene i hold 4 varierende symptomer på nedstemthed og bevægelsesforstyrrelser. Fire dyr måtte aflives umiddelbart. De øvrige dyr fik efterfølgende kontrolfoder i 2 dage, og ved aflivningen var ovennævnte kliniske symptomer forsvundet.

En enkelt han i hold 3 var stritpelset den første dag efter tildeling af testfoder. Hos én han blev observeret skorpedannelse omkring venstre øje i forperioden og under doseringsperioden.
I forbindelse med sektionen observeredes tegn på ændret adfærd af dyrene i hold 3. Sammenholdt med dyrene i hold 1 og 2 virkede dyrene i hold 3 mere aktive og følsomme for lyd, ligesom dyrene ikke på noget tidspunkt faldt til ro i burene. Dyrene var i en tilstand, der kan beskrives som "vedvarende explorativ". Der var tale om subjektive observationer, der ikke kan kvantiteres.

Legemsvægt og tilvækst for hanner og hunner var sammenlignelige holdene imellem.

Bortset fra en statistisk signifikant øget foderindtagelse for hanner i hold 3 i anden doseringsuge er data sammenlignelige holdene imellem.

Hold 3, hunner havde et statistisk signifikant nedsat foderforbrug i første, anden og fjerde doseringsuge. Relativt foderforbrug var kun statistisk signifikant lavere for hold 3, hunner i første doseringsuge.

For hunnerne i hold 3 var der et statistisk signifikant lavere vandforbrug og relativt vandforbrug i alle doseringsuger. Hunnerne i hold 2 udviste et lignende lavere vandforbrug og relativt vandforbrug i de to første doseringsuger.

Doseringen medførte følgende statistisk signifikante effekter på følgende plasma parametre:
Cholinesterase: Hunner, hold 2 havde lavere niveau end hold 1.
Kreatinin: Hunner, hold 3 havde lavere niveau end hold 1.
Glucose: Hunner, hold 3 havde lavere niveau end hold 1.

Doseringen medførte ikke nogen statistisk signifikant effekt på følgende plasma parametre for han og hun rotter:
ALAT, albumin, alkalisk fosfatase, kalcium, cholesterol, protein og urea.

Doseringen medførte følgende statistisk signifikante effekter for hjerne cholinesterase / hjerne protein:
Hunner: Hold 2 og 3 har et dosis afhængigt lavere niveau end hold 1. I hanner ses et tilsvarende dosis afhængigt fald, men effekten er ikke signifikant (se tabel A1).

Hanrotter i hold 2 og 3 viste et statistisk significant ($p<0,05$) lavere gennemsnit for WBC i sammenligning med kontrolholdet. Ved differentialtælling fandtes gennemsnit for neutrofile granulocyter statistisk signifikant ($p<0,05$) højere for hold 3 i sammenligning med kontrolholdet. En lignende tendens observeredes for hunrotter, dog uden statistisk signifikans (se tabel A2).

Ved obduktionen fandtes ingen makroskopiske forandringer af 4 hunrotter i hold 4 aflivet efter 1 døgns dosering. De øvrige han og hunrotter i hold 4 blev aflivet 4 dage efter forsøgets start. Disse blev ikke obduceret.
Ved obduktion af han og hunrotter i hold 1, 2, og 3 fandtes i forskellige organer tilfældige blødninger, som normalt kan observeres i forbindelse med bedøvelse med kuldioxid. De øvrige få forandringer skønnes tilfældige og uden sammenhæng med behandlingen.

Behandling medførte en statistisk signifikant forøgelse af både den absolutte ($p<0,05$) og relative ($p<0,001$) levervægt for han- og hunrotter i hold 3 (se tabel A3). For de øvrige organer konstateredes ingen effekt af behandlingen.

Ved den histopatologiske undersøgelse af 4 hunrotter aflivet efter 1 døgns dosering fandtes i leveren moderat diffus single cell degeneration/nekrose samt hyperchrome nerveceller i storhjernen. Disse forandringer, som skønnes relateret til behandlingen, blev ikke observeret i nogle af de øvrige dyr aflivet ved forsøgets afslutning.

Hos de øvrige han- og hunrotter, som blev aflivet ved forsøgets afslutning, observeredes få og tilfældige histopatologiske forandringer i hold 1 og hold 3. Ingen af forandringerne i hold 3 skønnedes at være relateret til behandlingen, hvorfor histopatologisk undersøgelse af hold 2 ikke blev foretaget.

Med forbehold for de variationer, der kan være i forsøgsbetingelserne mellem den aktuelle undersøgelse og de undersøgelser, der ligger til grund for de valgte koncentrationer i foderet i det aktuelle forsøg, viser resultaterne, at samtidig eksponering for en blanding af pesticider resulterer i en toksisk effekt i forsøgsdyr, der er forskellig fra den, man ser ved eksponering for de enkelte pesticider i blandingen. For nogle af de målte parametre kunne den observerede effekt tyde på, at der er tale om en additiv effekt af stofferne i den aktuelle blanding. Ifølge litteraturen er en sådan additiv effekt ikke overraskende (Groten et al 1997 & Jett et al 1999). Vedrørende den påviste hæmning af cholinesterasen og dennes mulige indflydelse på de observerede adfærdsændringer må vurderingen af en eventuel samspilseffekt afvente en tilsvarende undersøgelse af CH alene samt eventuelt en undersøgelse af AL og AL og CH i blanding i IFT's dyremodel.

2 Formål

Forforsøg til undersøgelse af mulig samspilseffekt ved samtidig eksponering af rotter for pesticider, der kan optræde som rester i levnedsmidler, i deres foder i 28 dage. Undersøgelsen gennemført efter OECD test guideline nummer 407 (OECD TG 407) med reduceret antal parametre og efter principperne for OECD GLP.

3 Forsøgsdesign

Dyrene blev eksponeret for en kombination af pesticider via foder i overensstemmelse med OECD TG 407 med følgende afvigelser:

Udvidet observation, som foreskrevet i OECD TG 407, med henblik på undersøgelser for ændret adfærd blev ikke foretaget udover udvidet observation af dyrene uden for burene i forbindelse med vejning af dyrene og ved aflivningen.

Analyse for natrium og kalium i plasma blev ikke foretaget. I stedet for blev der analyseret for Calcium og cholinesterase aktivitet i plasma og cholinesterase aktivitet og protein i hjernehomogenat.

4 Materialer og metoder

4.1 Test- og kontrolstof

Chlorpyrifos (CH), CAS nummer 002921-88-2.Bromopropylat (BR), GS19851A, technical, P.408159, 92.50% (Novartis).
Alfacypermethrin (AL), CAS nummer 67375-30-8. Carbendazim (CB), technical, Hoe 017411 00 ZD99 0028 (AgrEvo code).
Mancozeb (MA), CAS nummer 8018-01-7.

CH , BR, AL, CB og MA blev samlet iblandet pulveriseret chowfoder (Altromin), der blev slået til pellets, i koncentrationer, der for de enkelte stoffer svarede til 1/5 af NOAEL, NOAEL og 5 gange NOAEL (NOAEL er den højeste dosis for de respektive stoffer fundet i 90-dagesforsøg i rotter, der ikke gav toksikologisk effekt (Kilde er JMPR-monografier (BR)samt MST-vurderinger). Ved håndtering af teststoffer herunder ved fremstilling af foder blev det pålagt at bære handsker og støvmaske. Det blev foreskrevet at foderblanding skal foregå under udsugning m.h.p. at reducere udsættelse for støvpartikler.
Foderet blev fremstillet af Altromin International, Tyskland. Tetstofferne opbevares på køl hos Altromin International, Tyskland. Relavante dokumenter vedr. sikerhedsforanstaltninger ved håndtering samt specifikation mm., der blev modtaget sammen med de enkelte teststoffer blev medsendt.

4.2 Dyr

Dyreart, stamme og sygdomsstatus: Rotte/ MOL: Wist (Hannover), SPF. (Begrundelse: Instituttet har erfaring med denne Wistar rotte).

Antal: 48
Køn: 24 han og 24 hun.

Alder/vægt ved ankomst: Ca. 4 uger **(**ca 45-55 gram. Vægten 3 dage efter ankomst lå imellem 60g og 73g for hannerne og imellem 53g og 66g for hunnerne**)**

Leverandør: M & B A/S, Tornbjergvej 40, Ejby, 4623 Ll. Skensved, Danmark.

Dyreværn/-etik: Se under 6.7.

4.3 Modtagelse, randomisering og mærkning af dyr

Dyrene blev randomiseret på hold (jf. holdopstilling), 6 dyr/køn/hold, således at den gennemsnitlige legemsvægt og spredning var sammenlignelige holdene imellem (IT/DYR/GNAV/08). Dyrene blev øremærket i henhold til IT/DYR/GNAV/011. Desuden blev rotten med det højeste dyrenummer i hvert bur mærket med et tusch mærke på halen.

4.4 Opstaldning af forsøgsdyr

Rumnr.: E 220

Burtype: Trådbure (de første dage dog i makrolombure) type III med træklods, 2 dyr af samme køn pr. bur.
Miljø: Rumtemperatur blev sat til 21 ± 1°C.
Luftfugtighed blev sat til 55 ± 5%
Lys fra kl. 21 til 09. Mørke fra kl. 09 til 21.
Luftskifte 10 /t.

Der er konstateret afvigelser i luftfugtigheden d. 6/3, 8/3 og 15/3 1999. I alle tilfælde var der tale om en kortvarig overskridelse, og der er ikke målt luftfugtighed på over ca. 80%.
Det vurderes, at de konstaterede afvigelser ikke har haft indflydelse på undersøgelsens resultat.

4.5 Foder/fodringsmønster

Altromin 1324, vedligeholdelsesfoder. Der tildeles foder ad libitum.

4.6 Vand

Syrnet drikkevand ad libitum (IT/DYR/GENE/024)

4.7 Holdinddeling og dosering

Dosering via foder.

Hold 1 (Dyr nr. 1-6/7-12, han/hun): Kontrol (Altromin 1324) med vehikel (acetone)

Hold 2 (Dyr nr. 13-18/19-24, han/hun) 1/5 x NOAEL: mg/kg foder: [CH; 0.3, BR; 60, AL; 36, CB; 90 & MA; 25], dvs totalt 211.3 mg/kg foder.

Hold 3 (Dyr nr. 25-30/31-36, han/hun): NOAEL: mg/kg foder: [CH ; 1.5, BR; 300, AL; 180, CB; 450 & MA; 125], dvs totalt 1056.5 mg/kg foder.

Hold 4 (Dyr nr. 37-42/43-48, han/hun): 5 x NOAEL: mg/kg foder: [CH ; 7.5, BR; 1500, AL; 900, CB; 2250 & MA; 625], d.v.s. totalt 5282.5 mg/kg foder.

(Hold 4 udgik af forsøget efter blot én dags dosering.)

Kontrol af dosering/stofforbrug: Der blev udtaget foderprøver (ca. 50 g) af test-foderblandinger (IT/DYR/FODER/016) ved doseringens start og afslutning. Prøverne blev sendt til afdelingen for kemiske forureninger ved Institut for Fødevareundersøgelser og Ernæring, VFD, og analyseret for koncentration og opblanding (homogenitet) samt holdbarhed/stabilitet ved stuetemperatur og ved køleskabstemperatur af ovennævnte pesticider i de respektive foderblandinger.

Resultaterne af analyserne viste, at koncentrationerne af pesticiderne i de tre test-foderblandinger var som planlagte på 2 nær undtagelser (Genfinding på

76 - 107%). Den ene undtagelse gælder Chlorpyrifos i hold 2, der lå under den planlagte koncentration (genfinding på 61%) og Carbendazim i hold 4, der var højere end planlagt (genfinding på 122%).
Test for homogenitet foretaget på hold 2-foder viser, at opblandingen af pesticiderne var tilfredsstillende.
Undersøgelserne for holdbarhed af teststofferne i foderblandingen viste tilfredsstillende holdbarhed for alle stoffer på nær Mancozeb, der var mindre stabilt ved stuetemperatur. Dette sidste må inkluderes ved vurderingen af eksponeringen af rotterne, da foderet blev givet til rotterne for en uge ad gangen..

4.8 Biologiske parametre

Kliniske observationer: Dyrene blev tilset 2 gange dagligt i forsøgsperioden i henhold til IT/DYR/GENE/023. Kliniske fund noteredes på tilsynsskema med angivelse af dato for observationen og dyre- og forsøgsnummer.

Udvidet klinisk observation: Første gang i forperiode og derefter ugentligt.

Adfærdstestning: Blev ikke foretaget.

Øjenundersøgelse: Blev ikke foretaget.

Legemsvægt, tilvækst, foderforbrug og vandforbrug: Dyrene blev vejet ved forsøgsstart, derefter hver syvende dag og på aflivningsdagen (IT/DYR/DATA/04). Tilvæksten blev beregnet én gang ugentligt. Tildeling af foder, tilbagevejning af restfoder og måling af vandforbrug blev foretaget en gang ugentligt på de samme dage som dyrene blev vejet.

4.9 Blodprøvetagning

Plan for blodprøvetagning:

Opsamling af blod (IT/DYR/GNA/012) til hæmatologi og klinisk kemi blev foretaget .

4.10 Urinopsamling

Indikation (øget vandforbrug eller farvet urin) ved slutningen af doseringsperiode. Undersøgelse af urin blev ikke foretaget.

4.11 Klinisk kemiske parametre

Blod og hjernehomogenat: Plasma analyseres for flg. parametre: glukose (IT/MUT/KLK/16), cholesterol (IT/MUT/KLK/20), urea (IT/MUT/KLK/21), kreatinin (IT/MUT/KLK/18), calcium (IT/MUT/KLK/17), albumin (IT/MUT/KLK/31), protein (IT/MUT/KLK/19), cholinesterase (IT/MUT/KLK/23), alanin-amino-transferase (IT/MUT/KLK/25), alkalisk fosfatase (IT/MUT/KLK/24). Hjernehomogenat fra venstre hjernehalvdel analyseres for protein (IT/MUT/KLK/19) og cholinesterase (IT/MUT/KLK/23).

Urin: Urin analyseres efter indikation ifølge IFT/MUT/KLK/11.

4.12 Hæmatologiske parametre

Følgende parametre blev bestemt: Middelcellevolumen, hæmoglobin koncentration, antal røde og hvide blodlegmer, antal thrombocyter, differentialtælling af leukocytter. Efterfølgende beregnedes hæmatocrit, "mean cell hemoglobin concentration", "mean cell hemoglobin" og "mean cell volume" (IT/PAT/APP/01 og IT/PAT/PROC/12).

Koaguleringstid*:* Udførtes ikke.

4.13 Morfologiske undersøgelser af urin

Undersøgelse blev ikke gennemført.

4.14 Patologiske parametre

Aflivningskriterier: Klinisk syge dyr blev sendt til sektion, når tilstanden skønnedes at medføre døden inden for 24 timer, eller hvis dyrets tilstand var sådan, at det måtte anses for uforsvarligt eller uetisk at lade dyret leve (f.eks. længerevarende ophørt ædelyst eller store vægttab).

Nødsektion: Aflivning foretages i henhold til IT/PAT/PROC/01. Selvdøde dyr kommes i en plastikpose og bringes hurtigst muligt på køl, (kølelab. F 206), indtil obduktion kan finde sted, og patologisk sektion/vagthavende dyrlæge underrettes. Ved dødsfald eller aflivning før den planmæssige aflivning håndteres dyret i henhold til IT/PAT/PROC/03. Dyret obduceres i henhold til IT/PAT/PROC/02. Udtaget væv fikseres i 4% formaldehyd i henhold til IT/PAT/PROC/18.

Aflivnings- og sektionsprocedure :
Dyrene aflives som beskrevet i IT/PAT/PROC/01 og obduceres i henhold til IT/PAT/PROC/02, idet de nedenfor angivne væv henholdsvis vejes og fikseres.

Organer til vejning: Lever, nyrer, binyrer, testikler, epididymidis, thymus, milt, hjerne og hjerte fra alle hold trimmes og vejes (IT/PAT/PROC/09).

Følgende væv fikseres: Fra alle hold fikseres hjerne, hypofyse, oesophagus, spytkirtler (gl. mandibularis), mave, pancreas, duodenum, jejunum, ileum, caecum, colon, rectum, krøslymfeknude, lever, nyrer, binyrer, milt, hjerte, aorta, thymus, thyroidea/parathyroidea, trachea og lunger, testis, bitestis, ovarier, uterus, mælkekirtelvæv, accessoriske kønsorganer, urinblære, sternum (benmarv), rygmarv, perifer nerve (n. ischiaticus) og højre lårmuskel (højre kropshalvdel) (IT/PAT/PROC/18) samt alle makroskopiske forandringer.

Histologisk præparation**:** Fra hold 1 og 4 præpareres hjerne, hypofyse, krøslymfeknude, oesophagus, mave, pancreas, duodenum, jejunum, ileum, caecum, colon, rectum, lever, nyrer, binyrer, milt, hjerte, aorta, thymus, thyroidea/parathyroidea, trachea og lunger, testis, ovarier, uterus, urinblære, sternum (benmarv), perifer nerve (n. ichiaticus). Alle makroskopiske forandringer præpareres til histologisk undersøgelse.

Vævet trimmes, præpareres og snittes som anført i IT/PAT/PROC/04, 05, 06 og 07. På hjerne udføres præparation på højre halvdel. Vævssnittene farves som beskrevet i IT/PAT/PROC/19 med Meyers hæmatoxylin-eosin (HE-farvning). Hvis behandlingsrelaterede histologiske forandringer findes i hold 4

undersøges de(t) pågældende organ(er) fra hold 3. Finder der ligeledes forandringer i hold 3, undersøges de(t) pågældende organ fra hold 2.

Histopatologisk vurdering:
Hold 1 og 4 undersøges og undersøgelsen udvides på indikation til hold 3 og hold 2 i nævnte rækkefølge. Histopatologiske fund/diagnoser registreres i LABCAT (IT/PAT/DATA/03).

4.15 Statistiske metoder til behandling af data

Biologiske data:
Data for legemsvægt, foder- og vandforbrug blev overført fra vejeprogrammet til SAS, hvor data blev behandlet ved anvendelse af PROC GLM standardforskrift IT/BIOL/DATA/09 & IT//10. Data korrigeredes i henhold til de notater, der blev foretaget under den ugentlige kontrol af papirudskrifterne. Tilvækst, foderforbrug, vandforbrug, relativt foder- og vandforbrug blev beregnet. Der oprettedes tabeller med enkeltdyrsdata for legemsvægt og tilvækst, med enkeltbursdata for foder- og vandforbrug og tabeller med relativ foder og vandforbrug. Data for legemsvægt, foder- og vandforbrug blev analyseret med variansanalyse efterfulgt af Dunnetts test.

Biokemiske data:
Analyseredes i SAS efter IT/MUT/DATA/03. Students t-test blev anvendt.

Hæmatologiske og patologiske data:
Data analyseredes med variansanalyse og på indikation efterfulgt af Dunnetts test. Data der ikke opfyldte kriterier for variansanalyse eller Dunnetts test analyseres ved brug af Kruskal Wallis test.

4.16 Rapportering

Der blev udfærdiget en samlet rapport med bidrag fra de ansvarlige for biokemiske og patologiske parametre (Resultater fra foderanalysen vil medfølge som bilag til rapporten).

4.17 Arkivering

Følgende opbevares på Institut for Fødevaresikkerhed og Toksikologi i 10 år: Forsøgsplan, bilag og notater, "rådata", herunder håndskrevne og udprintede forsøgsresultater og anden dokumentation, beregnede data og statistiske behandling heraf, al modtagen og afsendt korrespondance, forsøgsrapport arkiveres i rum D 008. Fikseret væv opbevares i F 212 under forsøget og i F 007 efter, og paraffinindstøbt væv, histologiske præparater arkiveres i D 008. Foderprøve fra de 4 fodertyper opbevares 1 år i GLP-fryser rum D 008.

5 Resutater

5.1 Kliniske observationer

Efter 1 døgns dosering udviste dyrene i hold 4 varierende symptomer på nedstemthed og bevægelsesforstyrrelser. Fire dyr måtte aflives umiddelbart. De øvrige dyr fik efterfølgende kontrolfoder i 2 dage, og ved aflivningen var ovennævnte kliniske symptomer forsvundet.

En enkelt han i hold 3 var stritpelset den første dag efter tildeling af testfoder. Hos én han blev observeret skorpedannelse omkring venstre øje i forperioden og under doseringsperioden.
I forbindelse med sektionen observeredes tegn på ændret adfærd af dyrene i hold 3. Sammenholdt med dyrene i hold 1 og 2 virkede dyrene i hold 3 mere aktive og følsomme for lyd, ligesom dyrene ikke på noget tidspunkt faldt til ro i burene. Dyrene var i en tilstand, der kan beskrives som "vedvarende explorativ". Der var tale om subjektive observationer, der ikke kan kvantiteres.

I de øvrige hold var der intet at bemærke klinisk bortset fra en beskadigelse af højre øje hos en han i kontrolholdet som følge af blodprøvetagning.

5.2 Legemsvægt

Legemsvægten for hanner og hunner er sammenlignelige holdene imellem .

5.3 Tilvækst

Data for tilvækst for hanner og hunner er sammenlignelige holdene imellem.

5.4 Foderforbrug og relativt foderforbrug

Bortset fra en statistisk signifikant øget foderindtagelse for hanner i hold 3 i anden doseringsuge er data sammenlignelige holdene imellem. Tal for relativt foderforbrug er sammenlignelige for hannerne holdene imellem.

Hold 3, hunner havde et statistisk signifikant nedsat foderforbrug i første, anden og fjerde doseringsuge. Relativt foderforbrug er kun statistisk signifikant lavere for hold 3, hunner i første doseringsuge .

5.5 Vandforbrug og relativt vandforbrug

Data for hanner er sammenlignelige holdene imellem. For hunnerne i hold 3 var der et statistisk signifikant lavere vandforbrug og relativt vandforbrug i alle doseringsuger. Hunnerne i hold 2 udviste et lignende lavere vandforbrug og relativt vandforbrug i de to første doseringsuger.

5.6 Klinisk biokemi

Plasma analyser:

Doseringen medførte ikke nogen statistisk signifikant effekt på følgende plasma parametre for han og hun rotter:
ALAT, albumin, alkalisk fosfatase, kalcium, cholesterol, protein, urea.
Doseringen medførte følgende statistisk signifikante effekter på følgende plasma parametre:
Cholinesterase: Hunner, hold 2 havde lavere niveau end hold 1.
Kreatinin: Hunner, hold 3 havde lavere niveau end hold 1.
Glucose: Hunner, hold 3 havde lavere niveau end hold 1.

Hjerne analyser:

Doseringen medførte følgende statistisk signifikante effekter for hjerne cholinesterase / hjerne protein (se tabel A1).
Hunner: Hold 2 og 3 har et dosis afhængigt lavere niveau end hold 1.
I hanner ses et tilsvarende dosis afhængigt fald, men effekten er ikke signifikant.

A1. Relativ hjerne acetylsholinesterase aktivitet (hjerne acetylcholinesterase/ hjerne protein) hos hun- og han-rotter efter 28 dages exponering gennem foderet med hhv. hold 1: vehikel (acetone), hold 2: 1/5 NOAEL af AL, BR, CB, CH, MA og hold 3: 1 NOAEL af AL, BR, CB, CH, MA.

Køn		Hold		
		1	2	3
Han	Mean	346.97	340.85	316.81
	St.dv	62.43	48.33	16.60
Hun	Mean	344.00	316.07*	303.85*
	St .dv	29.45	14.67	12.52

*Statistisk signifikant forskelling fra hold 1 (P<0.05)

5.7 Hæmatologi

Doserede hanrotter i hold 2 og 3 viste et statistisk significant ($p<0,05$) lavere gennemsnit for WBC i sammenligning med kontrolholdet. Ved differentialtælling fandtes gennemsnit for neutrofile granulocyter statistisk signifikant ($p<0,05$) højere for hold 3 i sammenligning med kontrolholdet. En lignende tendens observeredes for hunrotter, dog uden statistisk signifikans (se tabel A2).

A2. Antal hvide blodlegemer (WBC, group mean ± st. dv) samt differential tælling (%, group mean ± st.dv) af hvideblodlegemer fordelt på lymfocytter, monocytter, netrofile-, eosinofile- og basofile granulocytter hos hun- og han-rotter efter 28 dages exponering gennem foderet med hhv. hold 1: vehikel (acetone), hold 2: 1/5 NOAEL af AL, BR, CB, CH, MA og hold 3: 1 NOAEL af AL, BR, CB, CH, MA.

	Hold	WBC x 10^9	Lymfocytter (%)	Monocytter (%)	Neutrofile (%)	Eosinofile (%)	Basofile (%)
Han	1	8.7 ± 3.5	87 ± 3	9 ± 4	4 ± 3	1 ± 0	0
	2	6.1 ± 1.1*	84 ± 4	11 ± 3	5 ± 2	0 ± 1	0
	3	5.1 ± 1.0*	82 ± 3	14 ± 3*	3 ± 1	1± 1	0
Hun	1	5.2 ± 1.8	85 ± 5	10 ± 4	4 ± 2	0 ± 1	0
	2	4.0 ± 0.6	85 ± 4	9 ± 4	5 ± 2	1 ± 1	0
	3	4.4 ± 1.2	88 ± 4	8 ± 4	3 ± 3	1 ± 1	0

*Statistisk signifikant forskelling fra hold 1 (P<0.05)

5.8 Patologi

Obduktionsfund Der fandtes ingen makroskopiske forandringer af 4 hunrotter i hold 4 aflivet efter 1 døgns dosering. De øvrige han -og hunrotter i hold 4 blev aflivet 4 dage efter forsøgets start. Disse blev ikke obduceret.
Ved obduktion af han -og hunrotter i hold 1, 2, og 3 fandtes i forskellige organer tilfældige blødninger, som normalt kan observeres i forbindelse med bedøvelse med kuldioxid. De øvrige få forandringer skønnes tilfældige og uden sammenhæng med behandlingen.
Den observerede forandring i hjernen hos hunrotte nummer 11, hold 1 diagnostiseret som "betændelse i hjernen" kunne ikke lokaliseres ved den senere histopatologiske undersøgelse.

Organvægte doseringen medførte en statistisk signifikant forøgelse af både den absolutte ($p<0,05$) og relative ($p<0,001$) levervægt for han- og hunrotter i hold 3 (se tabel A3). For de øvrige organer konstateredes ingen effekt af behandlingen.

A3. Legemsvægt, absolut og relativ levervægt hos hun- og han-rotter efter 28 dages exponering gennem foderet med hhv. hold 1: vehikel (acetone), hold 2: 1/5 NOAEL af AL, BR, CB, CH, MA og hold 3: 1 NOAEL af AL, BR, CB, CH, MA.

	Hold	Legemsvægt	Absolut levervæg (g)	Relativ levervægt (g/100 g legemsvægt)
Han	1	236.83 ± 14.39	9.05 ± 1.03	3.82 ± 0.25
	2	242.50 ± 23.54	8.85 ± 1.54	3.63 ± 0.30
	3	242.33 ± 12.69	11.03 ± 0.85*	4.55 ± 0.19**
Hun	1	163.50 ± 8.31	5.56 ± 0.53	3.40 ± 0.23
	2	165.67 ± 10.33	5.75 ± 0.30	3.48 ± 0.16
	3	159.67 ± 13.06	6.34 ± 0.62*	3.97 ±0.21**

*, **Statistisk signifikant forskelling fra hold 1 (*P<0.05, ** P<0.001)

Histopatologi Hos 4 hunrotter aflivet efter 1 døgns dosering fandtes i leveren moderat diffus single cell degeneration/nekrose samt hyperchrome nerveceller i storhjernen. Disse forandringer, som skønnes relateret til behandlingen, blev ikke observeret i nogle af de øvrige dyr aflivet ved forsøgets afslutning.

Hos de øvrige han- og hunrotter, som blev aflivet ved forsøgets afslutning, observeredes få og tilfældige histopatologiske forandringer i hold 1 og hold 3. Ingen af forandringerne i hold 3 skønnedes at være relateret til behandlingen, hvorfor histopatologisk undersøgelse af hold 2 ikke blev foretaget.

6 Diskussion

Undersøgelsens formål var at belyse mulige samspilseffekter ved samtidig eksponering af rotter for 5 pesticider, der kan optræder som rester i levnedsmidler. Dosisniveauernerne var sat ud fra oplysninger og vurdering af NOAEL af de enkelte stoffer baseret på JMPR monografi (BR, 1973) samt MST-vurderinger (CH, 1993, AL, 1996, CB, 1995 og MA, 1993). Dosis niveauer på 1/5 af NOAEL, NOAEL og 5x NOAEL af enkeltstofferne blev valgt udfra forventning om højst en mulig additiv effekt af de aktuelle stoffer i foderblandingen (Groten et al, 1997). Dog må der tages visse forbehold for diskussion af de fundne effekter i undersøgelsen i relation til data for effekt af de aktuelle enkeltstoffer. Det væsentligste forbehold for vurderingen af resultaterne er, at undersøgelserne, der ligger til grund for de valgte doser, er foretaget på andre laboratorier og hermed under forsøgsbetingelser, der ikke er identiske med forsøgsbetingelserne for denne undersøgelse . Af andre forhold skal nævnes, at vurderingen af enkeltstofferne er baseret på resumeer i monografier, og at de anvendte data stammer fra 90-dages undersøgelser, samt at der for BR er tale om oplysninger hentet fra en ældre monografi.

Undersøgelsesresultaterne viser, med de nævnte forbehold, at der var toksikologisk effekt af blandingen af de 5 pesticider, når de forekom i foderet i koncentrationer, der for de enkelte ligger på NOAEL. For en række fund gælder, at de forekom i det hold, der fik en femtedel af NOAEL for enkeltstofferne. Disse kritiske toksikologiske effekter var ændringer i blodbilledet og fald i cholinesterase i hjernen (cholinesterase / hjerne protein).

Ændringen i blodbilledet var kun statistisk signifikant i hannerne. Der er tale om et fald i antallet af hvide blodlegemer i hold 2 og 3 samt stigning af de neutrofile granolocyter. Det relative antal af lymfocyter udviser et ikke statistisk signifikant fald, der kan forklare faldet i det totale antal hvide blodlegemer. Ændringerne i blodbilledet i form af fald i lymfocyter og stigning i neutrofile granulocyter vurderes som en klassisk hæmatologisk stress reaktion (Ulich et al. 1978). Toksikologisk effekt i form af ændringer i blodbiledet er set i undersøgelser af AL i 90 dages undersøgelse i rotter sporadisk også ved NOAEL, og en ikke nærmere beskrevet effekt på blodbilledet er tillige rapporteret i 90 dages undersøgelse i rotter med MA. Den svage effekt på blodbilledet i hold 2 og 3 kan tolkes som en additiv effekt af de to aktuelle stoffer i foderblandingen.

Det eneste stof i den aktuelle foderblanding der vides at give anledning til hæmning af cholinesterase er CH. Koncentrationen i foderblandingerne var valgt udfra et NOAEL for cholinesterasehæmning i plasma og røde blodlegemer i et 90 dages fodringsforsøg i rotter. Cholinesterasehæmning i hjerne var i samme undersøgelse først påvirket ved en dosis, der var 10 gange højere. Effekten på plasma cholinesterase set i hold 2 hunner betragtes som et tilfældigt fund, men fund af ændringer i den tilsvarende hjerne parameter helt ned i den laveste dosering var ikke forventet, selvom der kun er tale om en statistisk signifikant effekt i hunnerne. En egentlig betydning af den aktuelle påvisning af hæmningen af cholinesterasen med henblik på vurderingen af en eventuel

samspilseffekt må afvente en tilsvarende undersøgelse af CH alene i IFT's dyremodel.
Undersøgelsen kan ikke fastslå, om den akut opståede effekt i dyrene i hold 4, symptomer på nedstemthed og bevægelsesforstyrrelser, herunder de histopatologiske forandringer i de 4 hunrotter, der blev aflivet efter et døgn, samt antydning i ændret adfærd i dyrene i hold 3 har forbindelse med cholinesterasehæmningen. En opfølgende undersøgelse af CH, som nævnt ovenfor vil eventuelt kunne afklare dette. Der foreligger oplysninger om, at AL er neurotoxisk. I specielle undersøgelser i rotter over 14 dage, hvor AL blev givet dagligt med sonde, er set ændringer i adfærd (ikke nærmere beskrevet) i doser ned til 20 mg/ kg lgv. Det nævnes, at der ses forandringer i biokemiske parametre, der tyder på, at der er tale om "axonal degenerering". Tillige angives det, at AL givet i foder til hunde over 13 uger i en koncentration på 270 ppm (svarende til ca. 7 mg/ kg lgv) gav anledning til adfærdsændringer i form af rystelser, ataxi og dårlig koordinering samt forhøjet temperatur. Der foreligger ikke oplysninger om, at de øvrige pesticider i foderblandingen er neurotoksiske eller giver anledning til ændringer i adfærd i øvrigt.

Den eneste forandring i patologiske parametre, som kan tilskrives doseringen, var en statistisk signifikant forøget levervægt (absolut såvel som relativ) for doserede hanner og – hunner i hold 3. Fundet vurderes at være en følge af en additiv virkning på leveren, der er målorgan i undersøgelser af 4 af de aktuelle pesticider, nemlig BR, AL, CB og MA.

Med forbehold for de variationer, der kan være i forsøgsbetingelserne mellem den aktuelle undersøgelse og de undersøgelser, der ligger til grund for de valgte koncentrationer i foderet i det aktuelle forsøg, viser resultaterne, at samtidig eksponering for en blanding af pesticider resulterer i en toksisk effekt i forsøgsdyr, der er forskellig fra den, man ser ved eksponering for de enkelte pesticider i blandingen. For nogle af de målte parametre kunne den observerede effekt tyde på, at der er tale om en additiv effekt af stofferne i den aktuelle blanding. Ifølge litteraturen er en sådan additiv effekt ikke overraskende (Groten et al. 1997 & Jett et al. 1999). Vedrørende den påviste hæmning af cholinesterasen og dennes mulige indflydelse på de observerede adfærdsændringer må vurderingen af en eventuel samspilseffekt afvente en tilsvarende undersøgelse af CH alene samt eventuelt en undersøgelse af AL og AL og CH i blanding i IFT's dyremodel.

7 Referencer

Miljøstyrelsen, Bekæmpelsesmiddelkontoret. Grundvurdering af Fastac indeholdende aktivstoffet alfacypermethrin; BILAG 1: Sagens oplysninger og Miljøstyrelsens vurdering. J.nr. M 741-0093, LLO/SM/11, Den 20 november 1996.

1973 Evaluations of some pesticide residues in food; Bromopropylate. WHO Pesticide Residues Series, No 3. World Health Organization, Geneva 1974.

Miljøstyrelsen, Bekæmpelsesmiddelkontoret. Grundvurdering af Derosal indeholdende Carbendazim som aktivstof. J.nr. M 741-0242, TBL/MKA/SM/sm/11, Den 4 januar 1995.

Miljøstyrelsen, kontor Bekæmpelsesmiddel. Vedrørende Empire. J.nr. M 741-1422, TBL/LK/jep/11, 18. marts 1993.

Miljøstyrelsen, kontor Bekæmpelsesmiddel. Vedrørende Dithane DG. J.nr. M 741-1097, TBL/LK/jep/11, 9. juni 1993.

Groten JP, Schoen ED, van Bladeren PJ, Frieke Kuber C, van Zorge JA, Feron VJ (1997): Subacute Toxicity of a Mixture of Nine Chemicals in Rats: Detecting Interactive Effects with a Fractionated Two-Level Factorial Design. Fundamental and Applied Toxicology **36**, 15-29.

Ulich TR, Keys M, Ni R-X, del Castillo J, Darkay EB (1988): The Contributions of Adrenal Hormones, Hemodynamic Factors, and the Endotoxin-related Stress Reaction to Stable Prostoglandin Analog-Induced Peripheral Lymphopenia and Neutrophilia. Journal of Leucocyte Biology **43**, pp. 5-10.

Jett DA, Navoa RV, Lyons Jr MA (1999): Additive inhibitatoryaction of chlorpyrifos and polycyclic aromatic hydrocarbons on acethylcholinesterase activity in vitro. Toxicology Letters 105, 223-229.

Appendix B

Dose-response study of Chlorpyrifos

(Study No. 01-06)

Absolute and relative organ weights, group mean results

B1. Absolute organ weights, group results male

		Group			
		1	2	3	4
Body Weight (g)	Mean	267.33	278.17	265.17	266.50
	St. dev.	24.51	12.27	26.03	14.68
Brain weight (g)	Mean	1.83	1.83	1.80	1.81
	St. dev.	0.15	0.05	0.07	0.06
Liver weight (g)	Mean	11.52	11.62	11.72	11.55
	St. dev.	1.35	0.65	1.41	0.95
Paired kidney (g)	Mean	1.85	1.83	1.85	1.76
	St. dev.	0.14	0.10	0.16	0.16
Paired Adrenal (mg)	Mean	58.47	60.82	49.62	53.78
	St. dev.	5.49	4.70	8.84	6.37
Paired testis (g)	Mean	3.01	2.99	2.98	2.96
	St. dev.	0.29	0.17	0.14	0.16
Thymus Weight (g)	Mean	0.55	0.56	0.53	0.49
	St. dev.	0.09	0.11	0.11	0.04

B2. Relative organ weights (absolute organ weight/body weight x 100), group results male

		Group			
		1	2	3	4
Relative Brain weight X 100	Mean	0.68	0.66	0.68	0.68
	St. dev.	0.02	0.04	0.05	0.03
Relative Liver weight X 100	Mean	4.30	4.18	4.42	4.34
	St. dev.	0.16	0.19	0.19	0.17
Relative Paired kidney X 100	Mean	0.69	0.66	0.70	0.66
	St. dev.	0.05	0.03	0.04	0.05
Relative Paired Adrenal x 1000	Mean	21.90	21.88	18.77	20.17
	St. dev.	1.56	2.01	3.30	2.21
Relative Paired testis X 100	Mean	1.13	1.07	1.13	1.11
	St. dev.	0.09	0.09	0.08	0.07
Relative Thymus Weight X 100	Mean	0.21	0.20	0.20	0.18
	St. dev.	0.04	0.05	0.03	0.02

B3. Absolute organ weights, group results female

		Group			
		1	2	3	4
Body Weight (g)	Mean	178.33	177.00	188.33	185.50
	St. dev.	12.53	6.72	5.54	13.22
Brain weight (g)	Mean	1.73	1.72	1.72	1.74
	St. dev.	0.10	0.11	0.06	0.06
Liver weight (g)	Mean	7.14	6.96	8.00	7.92
	St. dev.	0.77	0.55	1.28	0.63
Paired kidney (g)	Mean	1.27	1.16	1.26	1.31
	St. dev.	0.07	0.08	0.14	0.11
Paired Adrenal (mg)	Mean	70.53	68.45	65.18	78.90
	St. dev.	3.38	11.27	5.94	9.32
Thymus Weight (g)	Mean	0.41	0.38	0.45	0.43
	St. dev.	0.06	0.05	0.08	0.07

B4. Relative organ weights (absolute organ weight/body weight x 100), group results female

		Group			
		1	2	3	4
Relative Brain weight X 100	Mean	0.98	0.97	0.91	0.94
	St. dev.	0.11	0.07	0.05	0.04
Relative Liver weight X 100	Mean	4.01	3.93	4.25	4.27
	St. dev.	0.36	0.24	0.66	0.24
Relative Paired kidney X 100	Mean	0.71	0.65	0.67	0.71
	St. dev.	0.05	0.03	0.07	0.05
Relative Paired Adrenal x 10	Mean	39.72	38.68	34.72	42.65
	St. dev.	3.48	6.34	4.01	5.42
Relative Thymus Weight X 100	Mean	0.23	0.21	0.24	0.23
	St. dev.	0.02	0.02	0.04	0.03

Appendix C

Haematology, organ weights, macroscopic and microscopic pathology of the combined exposure study

(Study No. 02-22)

Haematology

Table 1C. Group results males, mean and standard deviation (st.dev) for White blood cell count (WBC), red blood cell count (RBC), haemoglobin (hb), Haematocrit (Hct), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelets (Plt).

		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
WBC	Mean	4.40	3.80	4.04	3.90	4.44	4.50
k/mm^3	St. dev.	1.29	0.56	1.10	0.78	0.94	1.13
RBC	Mean	7.46	7.31	7.01*	7.24	7,02*	7.12*
M/m^3	St. dev.	0.33	0.36	0.33	0.23	0.22	0.31
Hb	Mean	8.90	8.58	8.35*	8.53*	8.23*	8.34*
	St. dev.	0.25	0.37	0.32	0.20	0.21	0.27
Hct	Mean	43.21	41.26*	40.15*	41.06*	40.06*	40.35*
%	St. dev.	1.40	1.92	1.17	0.93	1.06	1.29
MCV	Mean	57.88	56.38	57.38	56.88	56.86	56.63
FL	St. dev.	2.17	0.52	2.33	1.36	1.95	1.51
MCH	Mean	1.19	1.17	1.19	1.18	1.18	1.18
Pg	St. dev.	0.04	0.01	0.06	0.02	0.04	0.03
MCHC	Mean	20.50	20.81*	20.76	20.80	20.59	20.70
G/dL	St. dev.	0.21	0.26	0.32	0.24	0.07	0.19
Plt	Mean	791.13	783.00	766.50	786.00	786.43	781.13
K/mm^3	St. dev.	81.98	75.69	62.41	37.04	89.30	67.89

*Significantly different from control (group 1), $P<0.05$

Table 2C. Differential count of white blood cells (%), lymphocytes (L), polymorphonuclear neutrophils (N), monocytes (M) and polymorphonuclear eosinophils (E). Group results male, mean and standard deviation (st. dev.)

		Group 1	Group 6
L	Mean	82.00	83.88
%	St. dev.	6.43	6.02
N	Mean	15.00	13.50
%	St. dev.	6.04	4.67
M	Mean	2.13	2.25
%	St. dev.	1.38	1.89
E	Mean	0.75	0.19
%	St. dev.	0.60	0.37
B	Mean	0	0
%	St. dev.	0	0
others	Mean	0.13	0.19
%	St. dev.	0.23	0.37

Table 3C. Group results females, mean and standard deviation (st.dev) for White blood cell count (WBC), red blood cell count (RBC), haemoglobin (hb), Haematocrit (Hct), Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelets (Plt).

		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
WBC	Mean	4.48	3.94	5.09	3.51	3.76	2.63*
k/mm^3	St. dev.	1.66	1.19	1.67	0.72	0.94	0.59
RBC	Mean	7.62	7.26	7.64	7.44	7.19*	7.11*
M/m^3	St. dev.	0.33	0.21	0.28	0.24	0.36	0.28
Hgb	Mean	8.93	8.50*	8.85	8.68*	8.43*	8.34*
	St. dev.	0.39	0.36	0.27	0.20	0.41	0.23
HCT	Mean	42.59	40.53*	41.83	40.88	39.84	39.59
%	St. dev.	1.65	1.60	1.13	0.97	2.06	0.84
MCV	Mean	55.88	55.63	54.63	55.13	55.50	55.88
FL	St. dev.	0.99	1.30	1.06	0.84	2.07	1.46
MCH	Mean	1.17	1.17	1.16	1.17	1.17	1.17
Pg	St. dev.	0.02	0.03	0.03	0.03	0.04	0.03
MCHC	Mean	20.94	21.01	21.15	21.20	21.14	21.11
G/dL	St. dev.	0.28	0.25	0.24	0.37	0.16	0.33
PLT	Mean	674.25	791.50	782.75	724.13	711.38	744.25
K/mm^3	St. dev.	114.41	99.29	72.14	147.75	156.78	49.64

*Significantly different from control (group 1), $P<0.05$

Table 4C. Differential count of white blood cells (%), lymphocytes (L), polymorphonuclear neutrophils (N), monocytes (M) and polymorphonuclear eosinophils (E). Group results females, mean and standard deviation (st. dev.)

		Group 1	Group 6
L	Mean	82.00	82.44
%	St. dev.	6.43	4.75
N	Mean	15.00	14.31
%	St. dev.	6.04	4.19
M	Mean	2.13	1.88
%	St. dev.	1.38	1.03
E	Mean	0.75	1.31
%	St. dev.	0.60	0.84
B	Mean	0	0
%	St. dev.	0	0
Others	Mean	0.13	0.06
%	St. dev.	0.23	0.18

Absolute and relative organ weights

Table 5C. Absolute organ weights (g) presented as mean and standard deviation, group results males

		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Body weight (g)	Mean	257.14	259.25	236.00	237.13	242.17	251.38
	St. dev.	19.81	14.93	19.11	15.03	12.50	13.80
Liver (g)	Mean	10.86	10.95	11.96	11.96	12.08	12.26
	St. dev.	1.40	0.89	1.42	0.98	1.11	0.89
R kidney (g)	Mean	0.89	0.91	0.92	0.88	0.92	0.92
	St. dev.	0.08	0.07	0.14	0.08	0.07	0.07
L kidney (g)	Mean	0.86	0.89	0.91	0.84	0.89	0.87
	St. dev.	0.06	0.08	0.13	0.08	0.06	0.07
R. adrenal (mg)	Mean	23.77	26.24	19.69	22.90	21.19	22.83
	St. dev.	7.02	4.52	8.21	3.86	2.99	2.38
L adrenal (mg)	Mean	27.73	29.30	24.25	23.13	24.64	23.71
	St. dev.	3.91	4.25	4.82	4.08	2.56	3.24
Spleen (g)	Mean	0.57	0.57	0.56	0.53	0.54	0.62
	St. dev.	0.06	0.06	0.15	0.06	0.10	0.07
Thymus (g)	Mean	0.58	0.49	0.40 *	0.40 *	0.43 *	0.43 *
	St. dev.	0.13	0.10	0.09	0.05	0.07	0.05
Thyroid (mg)	Mean	12.27	14.91	19.83 *	18.45	20.53 *	15.28 *
	St. dev.	1.09	2.95	7.61	8.95	9.18	2.94
R testes (g)	Mean	1.44	1.52	1.47	1.40	1.47	1.42
	St. dev.	0.03	0.10	0.10	0.07	0.14	0.26
L testes (g)	Mean	1.49	1.56	1.47	1.42	1.52	1.43
	St. dev.	0.05	0.11	0.10	0.07	0.19	0.26
R epidy-midis (g)	Mean	0.30	0.29	0.27	0.26	0.29	0.28
	St. dev.	0.02	0.04	0.05	0.02	0.02	0.05
L epidy-midis (g)	Mean	0.30	0.29	0.27	0.26	0.30	0.29
	St. dev.	0.03	0.03	0.05	0.02	0.03	0.05
Heart (g)	Mean	0.79	0.80	0.72	0.72	0.73	0.75
	St. dev.	0.10	0.05	0.07	0.05	0.05	0.05
Brain (g)	Mean	1.85	1.82	1.82	1.76	1.81	1.83
	St. dev.	0.08	0.10	0.07	0.07	0.06	0.07

*Significantly different from control (group 1), P<0.05.

Table 6C. Relative organ weights presented as (mean and standard deviation) x 100, group results males

		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Liver	Mean	4.22	4.22	5.06*	5.04*	4.95*	4.88*
	St. dev.	0.25	0.18	0.26	0.19	0.39	0.30
R kidney	Mean	0.35	0.35	0.39*	0.37	0.38	0.36
	St. dev.	0.01	0.02	0.03	0.03	0.03	0.01
L kidney	Mean	0.33	0.34	0.38*	0.35	0.37*	0.35
	St. dev.	0.02	0.02	0.03	0.02	0.02	0.01
R. adrenal	Mean	9.32	10.19	8.29	9.66	8.74	9.08
	St. dev.	2.81	2.09	3.18	1.55	1.60	0.84
L adrenal	Mean	10.78	11.37	10.22	9.74	10.30	9.43
	St. dev.	1.25	1.98	1.56	1.52	1.28	1.11
Spleen	Mean	0.22	0.22	0.24	0.22	0.22	0.25
	St. dev.	0.02	0.03	0.05	0.02	0.03	0.02
Thymus	Mean	0.22	0.19	0.17*	0.17*	0.18*	0.17*
	St. dev.	0.04	0.03	0.03	0.02	0.03	0.02
Thyroid	Mean	4.66	5.77*	8.45*	7.92*	8.56*	6.07*
	St. dev.	0.51	1.22	3.49	4.23	4.33	1.09
R testes	Mean	0.56	0.59	0.62	0.59	0.61	0.56
	St. dev.	0.05	0.06	0.05	0.05	0.07	0.10
L testes	Mean	0.58	0.60	0.63	0.60	0.63	0.57
	St. dev.	0.05	0.06	0.04	0.05	0.09	0.09
R epidy-midis	Mean	0.12	0.11	0.12	0.11	0.12	0.11
	St. dev.	0.01	0.01	0.01	0.01	0.01	0.02
L epidy-midis	Mean	0.12	0.11	0.12	0.11	0.12	0.12
	St. dev.	0.01	0.01	0.02	0.01	0.01	0.02
Heart	Mean	0.31	0.31	0.31	0.30	0.30	0.30
	St. dev.	0.02	0.02	0.02	0.01	0.02	0.01
Brain	Mean	0.72	0.70	0.77	0.75	0.75	0.73
	St. dev.	0.05	0.05	0.05	0.06	0.03	0.04

*Significantly different from control (group 1), P<0.05.

Table 7C. Absolute organ weights (g) presented as mean and standard deviation, group results females

		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Body Weight (g)	Mean	179.38	171.75	164.75	168.00	166.13	170.13
	St. dev.	19.65	12.78	7.70	9.70	9.06	11.52
Liver (g)	Mean	7.37	7.67	8.16	8.20	7.99	8.21
	St. dev.	0.98	0.91	0.75	0.51	0.66	0.45
R kidney (g)	Mean	0.66	0.65	0.63	0.60	0.64	0.65
	St. dev.	0.07	0.03	0.05	0.03	0.04	0.05
L kidney (g)	Mean	0.65	0.60	0.61	0.58	0.63	0.63
	St. dev.	0.06	0.02	0.05	0.04	0.07	0.06
R. adrenal (mg)	Mean	31.46	31.25	31.05	31.18	35.11	32.64
	St. dev.	4.11	8.15	3.98	3.28	10.55	4.57
L adrenal (mg)	Mean	33.25	34.20	31.13	35.38	36.68	34.57
	St. dev.	5.54	12.05	4.32	5.95	9.04	3.49
Spleen (g)	Mean	0.42	0.42	0.41	0.41	0.42	0.40
	St. dev.	0.03	0.07	0.08	0.05	0.05	0.04
Thymus (g)	Mean	0.47	0.45	0.35*	0.33*	0.38*	0.38*
	St. dev.	0.08	0.06	0.02	0.02	0.06	0.07
Thyroid (mg)	Mean	11.60	12.83	18.50*	18.46*	13.99*	15.14*
	St. dev.	2.70	3.13	8.35	5.44	2.03	3.80
R ovary (g)	Mean	0.05	0.05	0.05	0.04	0.06	0.05
	St. dev.	0.01	0.02	0.01	0.01	0.03	0.01
L ovary (g)	Mean	0.05	0.05	0.05	0.05	0.06	0.05
	St. dev.	0.01	0.01	0.01	0.01	0.03	0.01
Uterus (g)	Mean	0.40	0.41	0.36	0.39	0.35	0.36
	St. dev.	0.10	0.15	0.14	0.12	0.17	0.10
Heart (g)	Mean	0.60	0.60	0.55	0.57	0.56	0.59
	St. dev.	0.04	0.07	0.04	0.03	0.05	0.04
Brain (g)	Mean	1.71	1.68	1.72	1.71	1.71	1.72
	St. dev.	0.08	0.05	0.05	0.03	0.09	0.03

*Significantly different from control (group 1), P<0.05

Table 8C. Relative organ weights presented as (mean and standard deviation) x 100, group results females

		Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Liver	Mean	4.11	4.46(*)	4.94*	4.88*	4.81*	4.84*
	St. dev.	0.26	0.29	0.29	0.20	0.23	0.38
R kidney	Mean	0.37	0.38	0.38	0.36	0.39	0.39
	St. dev.	0.04	0.02	0.03	0.01	0.02	0.02
L kidney	Mean	0.37	0.35	0.37	0.35	0.38	0.37
	St. dev.	0.04	0.02	0.03	0.02	0.03	0.02
R. adrenal	Mean	17.66	18.11	18.84	18.56	21.09	19.50
	St. dev.	2.39	3.87	2.27	1.78	6.01	3.16
L adrenal	Mean	18.62	19.78	18.92	21.08	22.06	20.65
	St. dev.	3.00	5.98	2.72	3.60	5.17	2.70
Spleen	Mean	0.24	0.24	0.25	0.24	0.25	0.24
	St. dev.	0.02	0.02	0.04	0.03	0.02	0.02
Thymus	Mean	0.26	0.26	0.21*	0.20*	0.23(*)	0.22(*)
	St. dev.	0.03	0.04	0.02	0.02	0.04	0.04
Thyroid	Mean	6.65	7.42	11.45*	11.15*	8.40*	8.91*
	St. dev.	2.31	1.55	5.87	3.91	1.02	2.11
R ovary	Mean	0.03	0.03	0.03	0.03	0.04	0.03
	St. dev.	0.00	0.01	0.01	0.01	0.02	0.01
L ovary	Mean	0.02	0.03	0.03	0.03	0.04	0.03
	St. dev.	0.00	0.00	0.00	0.00	0.02	0.00
Uterus	Mean	0.22	0.24	0.22	0.23	0.21	0.21
	St. dev.	0.05	0.10	0.08	0.07	0.10	0.05
Heart	Mean	0.34	0.35	0.33	0.34	0.34	0.35
	St. dev.	0.03	0.03	0.02	0.01	0.03	0.02
Brain	Mean	0.96	0.98	1.04	1.02	1.03	1.02
	St. dev.	0.10	0.05	0.03	0.06	0.05	0.07

*Significantly different from control (group 1), P<0.05. (*) not significant by dunnetts

Table 9C. Microscopic findings in control (group 1) and highest dose group (group 6). N: Normal R: remarks (see list below).

Organ	Group 1 (male)	Group 6 (male)	Group 1 (female)	Group 6 (female)
Oesophagus	R	R	N	R
Stomach	N	R	N	N
Duodenum	N	N	N	N
Jejunum	R	N	R	N
Ilium	N	N	N	N
Caecum	N	N	N	N
Colon	N	N	N	R
Rectum	N	R	N	R
Trachea	N	N	R	R
Lungs	N	N	N	N
Kidneys	N	R	R	N
Liver	R	R	R	R
Adrenals	N	N	R	N
Pancreas	N	N	N	N
Thymus	R	R	N	N
Thyroid	N	R	R	R
Spleen	N	N	N	N
Lnn. Axilaris	R	N	R	N
Lnn. Mesenterialis	N	N	N	N
Urine bladder	N	N	N	N
Testes	N	N		
Epidymidis	N	R		
Vesicula seminalis incl. coagulation gland	N	N		
Prostate gland	N	R		
Vagina			R	R
Cerebrum	N	N	N	N
Cerebellum	N	N	N	N
Spinal cord	R	R	N	R
Heart incl. Aorta	N	N	N	N
Schiatic nerve	N	R	R	R

Bone marrow (sternum)	N	R	N	R

N: Normal R: Remarks see below

Remarks:

Oesophagus:
Group 1, male No 6: Tissue missing
Group 6, male No 164, female No 181, 182, 183: Tissue missing
Stomach:
Group 6, male no 164: Tissue missing
Jejunum:
Group 1, male No 6, 7 and female No. 17,18: One lymphoid follicle (peyer patch) present in lamina propria.
Group 6, female No. 182: One lymphoid follicle (peyer patch) present in lamina propria.
Colon:
Group 6, female No 177: 4 lymphoid nodules in lamina propria.
Rectum:
Group 6, male No 165: Tissue missing
Group 6, female No. 183: One lymphoid follicle in tela submucosa.
Trachea:
Group 1, female No. 21: Focus of lymphocyte infiltration under the epithelium
Group 6, female No. 182, 183: Tissue missing
Kidneys:
Group 1, female No 23: Pelvic inflammation grade 1
Group 6, male No165: Tubular cell regeneration
Liver:
Group 1 male No. 1, 2, 3, 5, 6, 7, 8: 1-3 foci of inflammatory cells
Group 6 male 161-168: Mild degree of centrilobular cell hypertrophia
Male No. 161, 162, 163, 165, 167, 168: 1-3 foci of inflammatory cells
Group 1 female No. 17, 19, 20, 21: 1-2 foci of inflammatory cells
Group 6 female No. 182, 183, 184: Mild degree of centrilobular cell hypertrophia
Female No. 177, 179, 181, 182, 183: 1-3 foci of inflammatory cells
Adrenal glands:
Group 1, female No. 17: Medulla missing in two sections
Thyroid gland:
Group 1, female No.24: Mild degree of follicular cell hypertrophy
Group 6, male No.161, 162, 163, 165, 166, 167, 168: Mild degree of follicular cell hypertrophy
Group 6, female No. 177, 178, 179, 180, 183, 184: Mild degree of follicular cell hypertrophy
Thymus:
Group 1 male No 2: Number of macrophages in cortex increased
Group 6 male No 164, 168: Number of macrophages in cortex increased
Lnn. Axillaris:
Group 1, male No. 8, female No 23: Tissue missing
Epidymidis:
Group 6, male No. 162: Decreased amount of sperm in both caput and cauda of right epidymidis
male No. 168: Vacuolarization of epithelium of Right epidymidis
Prostate gland:
Group 6, male No165: Inflammation in lateral prostate

Male No. 166: Tissue missing

Vagina:

Group 1, female No. 17, 20: Tissue missing

Group 6, female No. 179: Tissue missing

Sciatic nerve:

Group 1, female No 17, 23: Tissue missing

Group 6, male No. 165: Tissue missing

Group 6, female No. 178: Tissue missing

Spinal cord:

Group 1, male No. 6: Tissue of cervical region missing

Group 6, male No. 165: Tissue missing

Group 6, female No. 181: Tissue missing

No 180: Tissue of thoracal region missing

No 184: Tissue of lumbar region missing

Bone marrow:

Group 6, male No. 165: Tissue missing

Group 6, female No. 181: Tissue missing