Acta vet. scand. 1966, 7, 364—390.

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TOXICOLOGICAL STUDIES OF PHENOXYACETIC HERBICIDES IN ANIMALS *)

By

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The present study was initiated by the rather frequent occurrence during recent years in this country of cases of suspected, acute or chronic, poisoning by phenoxyacetic type herbicides in livestock or wildlife.

As active ingredients these materials usually contain 2,4-dichloro-, 2-methyl-4-chloro- or 2,4,5-trichloro-phenoxyacetic acids (denoted below as 2,4-D, MCPA and 2,4,5-T, respectively). Analogous phenoxypropionic and phenoxybutyric acids also are finding some use.

The acute toxicity of these compounds to animals is moderate. Their toxicology has been reviewed by *Rowe & Hymas* (1954) and *Dalgaard-Mikkelsen & Poulsen* (1962). Thus, acute oral LD_{50} values ranging between approximately 300 and 1000 mg/kg body weight have been reported for 2,4-D and for the other compounds in laboratory animals, and the toxicity to cattle and sheep appears to be of the same order. Dogs, however, seem to be more susceptible with LD_{50} values of about 100 mg/kg (*Drill & Hiratzka* 1953). No appreciable difference in toxicity has been observed between various esters and salts and between pure chemicals and formulated products (*Hill & Carlisle* 1947; *Rowe & Hymas*).

On repeated oral or parenteral administration several investigators have found phenoxyacetic acids to have a low chronic

*) Supported by a grant from "Albert Hjärrefonden".

toxicity in laboratory as well as in larger animals (see *Rowe & Hymas* and *Dalgaard-Mikkelsen & Poulsen*). In contrast, dogs might be severely poisoned after repeated doses as low as 20—25 mg/kg (*Drill & Hiratzka; Hill & Carlisle*). The low chronic toxicity of phenoxyacetic acids in cattle and sheep was confirmed in experiments by Palmer. An unexpected toxicity was found, however, in a trichlorophenoxypropionic ester (Silvex), a few doses of 100 mg/kg causing severe poisoning in cattle and sheep (*Palmer & Radeleff* 1964).

Feeding experiments with either treated crops, sprayed at varying rates with phenoxyacetic herbicides, or the chemical compounds in doses calculated to be equivalent to those encountered during field conditions, revealed no adverse effects in various farm animals (*Mitchell et al.* 1946; *Grigsby & Farwell* 1950; *Dalgaard-Mikkelsen et al.* 1959) or in laboratory animals (*Schillinger* 1960).

The symptoms of poisoning include locomotory disturbances, such as disinclination to move, stilted gait, muscular weakness and myotonia, and signs of irritation of the digestive tract. Occasionally, signs from the eyes and upper respiratory tract, such as conjunctivitis, nasal discharge and epistaxis, have been noted.

On autopsy no specific changes have been observed. Besides non-specific catarrhal lesions in the digestive tract, pneumonia has been noticed frequently and slight degenerative changes in the liver and kidneys occasionally. Erosions in the mouth and nasal passages have been observed in chronically poisoned cattle. Any histopathological explanation for the locomotory disturbances has not been obtained.

The experiments to be described below have been performed during several years with the purpose of studying the long-term effects of phenoxyacetic compounds in pigs, rats and chickens. In preliminary acute-toxicity studies calves were also included.

EXPERIMENTAL

Materials

2,4-D amine. Commercial formulation of the triethanolamine salt of 2,4-D, diluted with water to a concentration of 20 mg of 2,4-D per ml. The product used in the early single-dose experiments with pigs contained in addition 2 % of a wetting agent.

2,4-D K-Na salt. Aqueous solution prepared by dissolving the pure acid (m.p. 138—142°C) in a slight excess of aqueous KOH—NaOH

(1:1), adjusting pH to about 7 and diluting to a concentration of 20 mg of 2,4-D per ml.

2,4,5-T amine. Aqueous solution of the triethanolamine salt of 2,4,5-T, prepared by dissolving the pure acid (m.p. 155—157°C) in a slight excess of aqueous triethanolamine, adjusting pH to about 7 and diluting to a concentration of 20 mg of 2,4,5-T per ml.

2,4-D ester. Commercial formulation of the butyl ester of 2,4-D in a petroleum solvent, diluted, and emulsified, with water to a concentration of 20 mg of 2,4-D per ml.

Animals

Weight and age data refer to conditions at start of experiments.

Pigs, Swedish "Lantras" breed, raised at the institute, 18—25 kg, 8—12 weeks, castrated males, and females.

Sow, Swedish "Lantras" breed, 200 kg, 6 years.

Calves, SRB breed, 55—65 kg, 6—8 weeks, both sexes.

Albino rats, Sprague-Dawley, adult, 180—220 g, both sexes, and pregnant females, about 350 g.

Chicks, broiler, day-old, both sexes.

Chickens, White Leghorn breed, 0.8—1.0 kg, 8—10 weeks, both sexes, and New Hampshire breed, 2.2—2.9 kg, adult hens.

The pigs were maintained on a ration of ground barley supplemented with a commercial pig feed (Suggex, manufactured by AB Lactamin, Stockholm) and stirred with water to a thin slurry. The calves were given hay and a commercial feed supplement on dried-milk basis (Calvex, Lactamin). The chickens and rats were fed commercial pelleted feeds and had water *ad libitum.*

In the long-term experiments, controls, as nearly identical to the experimental animals in weight and age as possible and receiving the identical feed and treatment, were included.

All animals were kept in separate pens or cages, unless otherwise stated.

The animals were weighed bi-weekly. In the chicken experiments the number and weight of eggs produced were recorded.

Methods

Administration. In single-dose experiments animals were starved overnight before dosing. The materials under study were given by gastric intubation in short-term experiments, and mixed in feed or drinking water in long-term experiments. All doses were expressed in terms of the active ingredient.

Analytical methods. Routine blood analyses during the long-term experiments included determination of haematocrit, haemoglobin, plasma urea nitrogen and plasma glutamate-oxaloacetate-transaminase (GOT; *Karmen et al.* 1955) and, in special instances, differential cell counting and electrophoretic plasma protein fractionation with densitometric evaluation. Urine samples of pigs were tested for the

presence of protein, glucose, bilirubin, urobilin and acetone bodies. Unless otherwise indicated, the methods of *Hammarsten* (1955) were followed.

Phenoxy acids were determined in body fluids and tissues as described previously (*Erne* 1966 a), and plasma half-life of 2,4-D was estimated graphically (*cf. Erne* 1966 b).

Autopsy. At intervals during the long-term experiments an equal number of experimental and control animals were sacrificed, by exsanguination after anesthetizing with either mebumal sodium (in pigs) or chloroform (in rats and chickens), and subjected to autopsy within 15 min. after death. In addition, all animals dying or killed during the experiments because of a poor condition were autopsied. Organ weights were recorded throughout and the relative weights calculated.

Samples were regularly taken for *histological examination* from different parts of the following organs: digestive tract (salivary glands, oesophagus, stomach, small and large intestine, liver, pancreas), respiratory organs (trachea, lungs), excretory organs (kidneys), circulatory and haematopoietic systems (heart, vessels, lymph glands, spleen, bone marrow), nervous system (brain, spinal cord, peripheral nerves), sexual organs (testes, epididymides, prostate, ovaries, uterus), locomotory system (skeletal muscles and bones) and endocrine glands (hypophysis, epiphysis, thyroid, parathyroids, thymus, adrenals).

Fixatives and staining methods. Sections were ordinarily fixed in 10 % formaldehyde. For particular purposes Bouin's, Carnoy's and Helly's fluids were employed.

Frosen sections of the samples were stained with Scarlet Red for fat; the routine stains for paraffin sections were Ehrlich's haematoxylin and eosin, van Gieson and PAS.

RESULTS

A. *Calves*

Two calves were each given a single oral dose of 2,4-D K-Na salt (100 mg/kg). After subsiding of symptoms and a resting period of 4 weeks the animals were each given 2,4-D ester (100 mg/kg) and, after another 4-week resting period, 2,4-D amine (50 mg/kg) and then in the same way 2,4-D amine (first 100 and then 200 mg/kg).

With 2,4-D amine at 50 mg/kg, slight dysphagia, appearing on the second day and persisting for a few days, was noted in one animal; the plasma level of 2,4-D, after passing a maximum of about 100 µg/ml (at about 5 hours), dropped to below 10 µg/ml within 24 hours. With the ester no untoward effects were seen; the plasma level of 2,4-D remained low throughout the experiment. At 100 mg/kg, 2,4-D amine caused some anorexia, and in

one animal bloating, whereas the alkali salt caused dysphagia in one animal; in both instances peak plasma concentrations of about 150 µg 2,4-D/ml were attained, the values dropping to about 20 µg/ml within 24 hours after dosing. At the highest dosage level of 2,4-D amine, anorexia, thirst and muscular weakness, particularly of the hind quarters, were apparent in both animals for 3—4 days. In this case the maximum plasma level averaged 250 µg/ml and the value at 24 hours remained comparatively high (at about 70 µg/ml). The animals were not autopsied.

B. ***Pigs***

1. *Single dose*

Four groups of 2 pigs each were given 2,4-D amine as a single oral dose of 50, 100, 500 and 1000 mg/kg, respectively.

At 50 mg/kg, anorexia was seen in one animal, at 100 mg/kg also diarrhoea, a stilted gait and transitory depression. At the two higher dose levels signs of poisoning were severe, including vomiting, severe muscular weakness and general depression. Those animals were killed in a moribund state after 24 hours.

At about 5 hours after dosing, plasma 2,4-D in the first two groups attained average maximum levels of 120 and 230 µg/ml, respectively, the corresponding values for the other groups being 440 and 525 µg/ml, respectively. The average levels in the four groups at 24 hours were 35, 110, 400 and 580 µg/ml, respectively.

When single oral doses of 2,4-D ester (100 mg/kg) and 2,4,5-T amine (100 mg/kg) were given to 2 pigs each, the ester exerted no visible effects, whereas 2,4,5-T amine produced anorexia, vomiting, diarrhoea and locomotory disturbances in both animals. In the ester experiments plasma 2,4-D did not rise above 40 µg/ml. With 2,4,5-T, however, fairly high plasma levels were attained, the peak value being about 250 µg/ml and the 24 hours value about 80 µg/ml.

Surviving animals were killed 2—3 days after dosing. Autopsy of dead and killed animals revealed signs of gastro-intestinal irritation at all dose levels, at the two highest also accompanied by penumonia and renal and hepatic congestion.

2. *Short-term experiments*

2,4-D amine and 2,4-D ester were given as repeated oral doses to pigs according to the scheme of Table 1.

Table 1. Repeated oral administration of 2,4-D amine and ester to pigs. Short-term experiments.

Material	Animal no.	Dose mg/kg	Number of doses given	Duration, days	Clinical	Autopsy findings
2,4-D	4 B	50	3	5	No clinical signs	No significant gross changes
amine	8 A	50	8	18	During last week anorexia, diarrhoea, general depression	Ulceration of gastric mucosa, catarrhal enteritis
	4 C	50	10	15	No clinical signs	No significant gross changes
	4 A	50	15	20	„ „ „	Hyperplasia of bronchial lymph glands, focal fatty degeneration in kidneys
	11 A	50	51	103	During last 2 months anorexia and retarded growth	Catarrhal enteritis
	12 A	100	3	7	Vomiting, general depression	Ulceration of gastric mucosa, catarrhal colitis
	3 A	100	7	9	Vomiting, diarrhoea, muscular weakness	Ulceration of gastric mucosa, catarrhal enteritis, pneumonia, fatty degeneration in kidneys
	1 A	300	2	4	General depression	Fatty degeneration in kidneys
2,4-D	9 B	50	5	8	No clinical signs	No significant gross changes
ester	15 A	50	7	10	Anorexia, diarrhoea, muscular weakness	Catarrhal gastro-enteritis, pneumonia
	9 C	50	12	17	No clinical signs	No significant gross changes
	9 A	50	23	39	Muscular weakness	Hyperplasia of bronchial lymph glands, kidney enlargement
	2 A	300	3	6	Anorexia, diarrhoea, muscular weakness, general depression	Ulceration of gastric mucosa, catarrhal enteritis, pneumonia

As seen, the lesions were observed mainly in the digestive tract and the respiratory and excretory organs. The stomach ulcers were consistently located in the fundus region of the stomach. In cases with macroscopic pneumonia the histopathological picture was dominated by alveolar cell proliferation. The focal fatty degeneration of the kidneys involved varying tubular sections and Henle's loop.

In those animals which developed definite signs of poisoning after repeated daily dosing with 2,4-D persistently high plasma levels were found. The levels usually remained in the region of 200—400 μg/ml even at 24 hours after a dose. As a contrast, animals tolerating the repeated administration of 50 mg/kg/day without clinical effects were seen to eliminate the phenoxy acid at an enhanced rate. After an adaptation period the plasma levels in these animals regularly declined to about 10 μg/ml within 24 hours after dosing.

3. *Long-term experiments*

a) *Young pigs.* Five litter mates (3 castrated males and 2 females, 8 weeks old), kept in a common pen throughout the experiment, were fed 2,4-D amine at 500 p.p.m. in a standard feed for up to 12 months. A group of normal pigs of the same age served as controls.

No indigestion was seen during the experiment. The experimental animals completely consumed their daily feed ration although slower than the controls. The growth rate was irregular and depressed (Fig. 1).

After about one month 3 animals developed locomotory disturbances, in the form of a stilted gait, which gradually increased in severity. Two of the animals showed strongly elongated lateral hoofs on the forelegs (Fig. 2), a rather unique phenomenon in growing pigs and not seen in the controls despite of marked locomotory disturbances.

The animals were killed after 2—12 months. The relative organ weights (heart, spleen, liver, kidney) were within the normal range. Gross pathological changes were not noted. Clinical-chemical and histopathological results are reported below together with those of experiment c).

b) *Pregnant sow.* One sow, which had previously farrowed five times without complications, was fed 2,4-D amine at the level of 500 p.p.m. in standard feed during her entire seventh

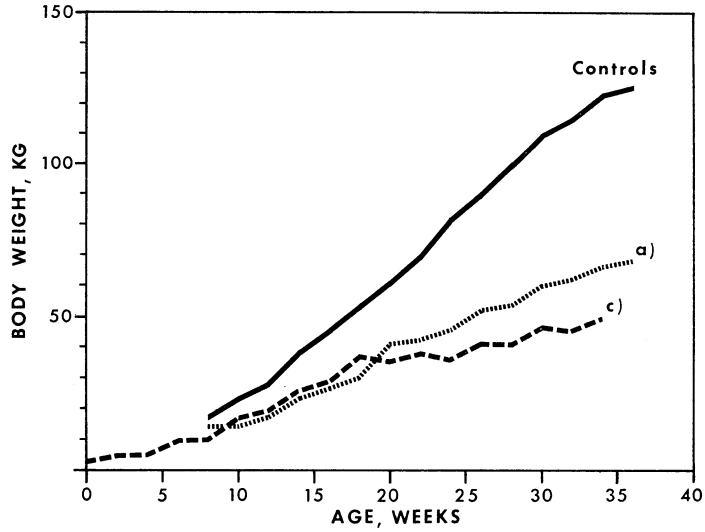


Figure 1. Growth rate of pigs continuously given 2,4-D (500 p.p.m.) in feed. Experimental groups a) and c) (see text).

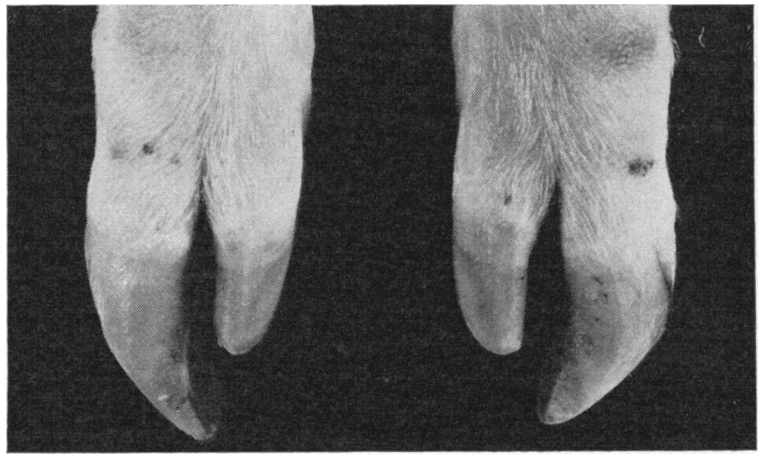


Figure 2. Foreleg of pig given 2,4-D (500 p.p.m.) in feed, experiment a). Elongated lateral hoofs.

pregnancy and for 6 further weeks. Anorexia was apparent throughout the experiment. Indigestion or other signs of illness were not seen. During a protracted parturition, one mumified foetus and 15 piglets, with weights ranging from 380 to 1090 g were delivered. The piglets were clearly underdeveloped and apathetic and did not willingly suck. Ten of the piglets (2 males

and 8 females) died during the first day. The autopsy findings were those of a generalized anaemia. On histopathological examination, embryonic haematopoietic foci were noticed in the livers. No malformations were observed. 2,4-D was found in livers, kidneys and lungs of the dead piglets at concentrations of 15 to 80 μg/g. The survivors (4 males and 1 female) were subnormal in weight and anaemic. On the fifth day they received customary iron supplementation intramuscularly (Grisofer, Astra, 150 mg Fe per animal).

After parturition the sow was unable to rise unaided. The condition improved on tocopherol injection, but locomotory disturbances persisted. Six weeks after parturition the sow developed a sudden lameness of one hind leg with consequent complete inability to rise and was slaughtered. Except for excessive disc degenerations and protrusions and discospondylitis in posterior thoracic and lumbar regions of the spine, no gross changes were seen.

c) *Second generation.* The surviving 5 piglets of the sow in experiment b) were reared artificially after the death of the sow, on a pelleted feed containing 2,4-D amine at the level of 500 p.p.m.. From two months of age they received the feed used in experiment a), above. The males were castrated according to current practice.

The feed was consumed reluctantly, but no indigestion was seen during the experiment. The growth was retarded as compared with controls (Fig. 1).

Locomotory disturbances similar to those seen in experiment a), above, although less severe, developed during the third month. The length of the hoofs remained normal throughout the experiment, although fissures and ulcerations were seen on the abaxial surface of the wall.

As seen from Table 2, summarizing some of the clinical-chemical data of experiments a) and c), the haematocrit and haemoglobin values were lowered in both experimental groups as compared to controls. (Differential cell-counting, however, did not reveal any abnormalities.) GOT values were slightly elevated and the albumin levels and albumin-globulin ratios possibly somewhat reduced. Plasma protein and urea nitrogen were not significantly altered. The plasma half-life of 2,4-D was highly variable and slightly elevated when compared with the normal value of 12 ± 2 hrs. obtained previously (*Erne* 1966 b).

Table 2. Clinical-chemical data (m ± s) for pigs given 2,4-D (500 p.p.m.) continuously in feed. Experimental groups a) and c) (see text).

Group	Haematocrit %	Haemoglobin g/100 ml	Plasma Urea N mg/100 ml	Plasma GOT Karmen Units	Plasma protein g/100 ml	Albumin g/100 ml	Albumin - globulin ratio	2,4-D half life (plasma), hrs.
				One month				
a)	35 ± 5	9.5 ± 1.2	12.2 ± 3.6	54 ± 9	—	—	—	19 ± 5
c)	29 ± 4	9.0 ± 1.5	11.3 ± 3.8	39 ± 7	—	—	—	—
Controls	43 ± 3	11.8 ± 0.4	10.1 ± 1.2	28 ± 8	—	—	—	—
				Three months				
a)	40 ± 4	12.3 ± 1.2	—	36 ± 9	—	—	—	16 ± 11
c)	31 ± 2	12.2 ± 0.7	—	42 ± 4	—	—	—	—
Controls	46 ± 2	15.1 ± 0.5	—	26 ± 5	—	—	—	—
				Six months				
a)	41 ± 4	12.8 ± 0.8	13.6 ± 2.7	48 ± 11	6.6 ± 0.3	2.8 ± 0.3	0.72 ± 0.05	17 ± 9
c)	40 ± 4	13.1 ± 1.3	12.4 ± 4.1	46 ± 12	6.7 ± 0.7	2.3 ± 0.3	0.57 ± 0.10	24 ± 8
Controls	49 ± 2	16.6 ± 0.5	9.1 ± 1.7	24 ± 2	7.6 ± 0.3	3.8 ± 0.2	0.90 ± 0.05	—

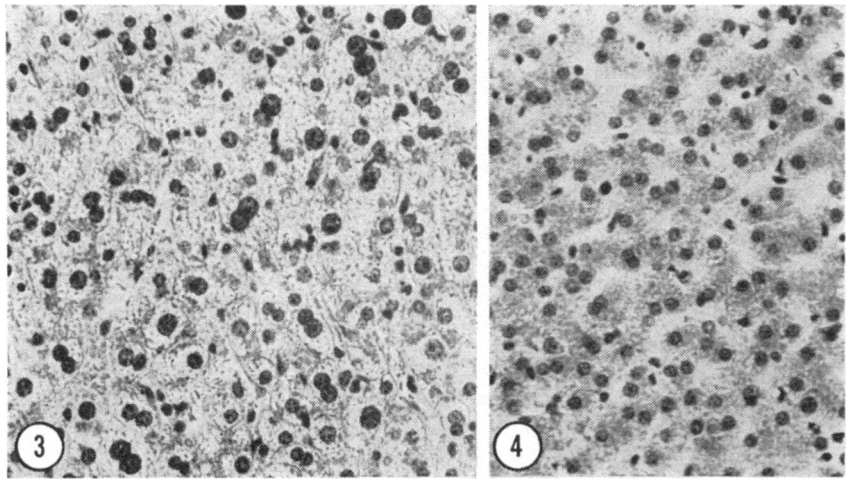


Figure 3. Liver of pig given 2,4-D (500 p.p.m.) in feed, experiment c). Parenchymatous degeneration and nuclear hypertrophy in liver cells. Scarlet Red, 240 ×.

Figure 4. Liver of normal pig.

In addition, albuminuria was common in both experimental groups.

The animals were killed at the age of 7—8 months and autopsied. The relative organ weights were within the normal range.

No unequivocal gross changes were observed in this experiment, except for multiple disc degenerations in the cervical and lumbar spine regions in 2 animals.

On histological examination of the pigs from both experiments parenchymatous degeneration with abnormally varying nuclear size of hepatic cells was apparent, particularly in experiment c). In many cells one or two giant nuclei were seen (Figs. 3 and 4). In the kidneys the only detectable changes were slight focal dilatation of varying tubular sections, often accompanied by slight degeneration of epithelial cells. In one animal, changes were also apparent in the spinal cord, as multiple sclerotic foci in the lateral columns of the white substance. Significant lesions were not found in other organs.

C. *Rats*

1. *Single dose*

Twenty-five male and 10 female albino rats, weighing about 200 g, were given a single oral dose of 2,4-D amine (100 mg/kg), in connection with distribution studies (*Erne* 1966 b). Likewise, three groups of male rats each were given 2,4-D potassium-sodiumsalt, 2,4-D butylester and 2,4,5-T amine, respectively, as single oral doses of 100 mg/kg.

The animals were killed at intervals during 2 to 72 hours. No clinical effects or gross pathological changes were seen. With 2,4-D amine average maximum plasma levels of about 180 µg/ml for the males and about 100 µg/ml for the females were attained. In both instances the levels dropped to about 1 µg/ml within 24 hours. Similar results were obtained with the alkali salt and with 2,4,5-T amine. Administration of the ester, on the other hand, resulted in only low plasma levels of 2,4-D, the average maximum value being 20 µg/ml.

2. *Long-term experiments*

a) *Pregnant rats.* Ten newly mated, female rats, weighing about 350 g, were evenly distributed between one experimental and one control group. The experimental animals received 1000 p.p.m. of 2,4-D in the drinking water during the pregnancy and for further 10 months, otherwise feed and treatment of the groups were identical. No significant effects on the course of the pregnancy were noted, and parturition proceeded normally. The average number of young rats per litter was 6 for the experimental and 5 for the control group. No malformations were observed. In the first-generation rats no clinical signs or distinct morphological changes were seen throughout the experiment.

b) *Second generation.* After weaning, the young rats from experiment a) (experimentals: 10 males and 12 females; controls: 9 males and 8 females) were caged in groups of 3 or 4 and the administration of 2,4-D (1000 p.p.m.) continued for up to 2 years.

The experimental animals showed a reduced intake of feed and water as compared with the controls, and an attendant depression of the growth rate (Fig. 5). Temporarily also diarrhoea and a poor general condition were apparent. Clinical-chemically (haematocrit, haemoglobin, plasma GOT) there were no signi-

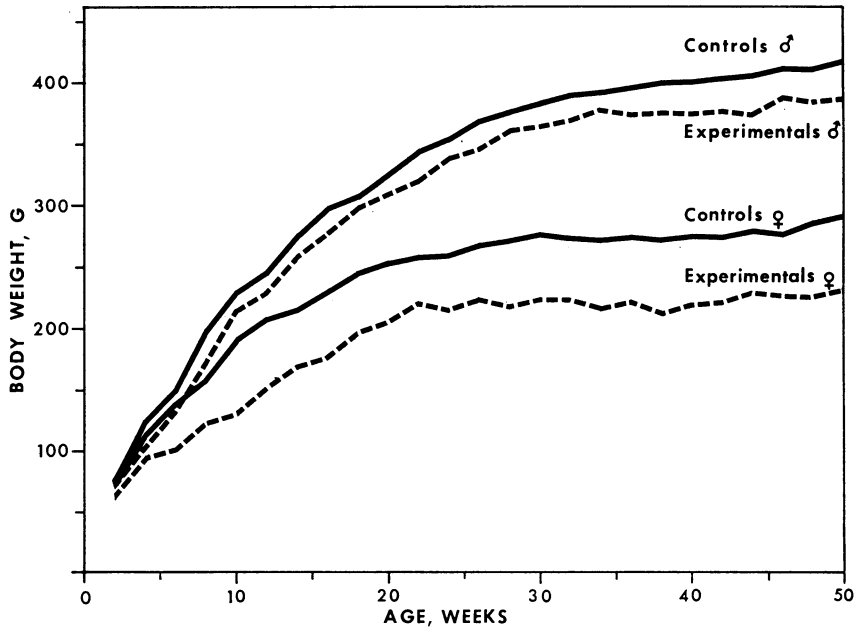


Figure 5. Growth rate of rats continuously given 2,4-D (1000 p.p.m.) in drinking water, experiment b) (see text).

ficant differences between the experimental and control groups. The plasma elimination rate of 2,4-D was normal, the half-life consistently being about 3 hours. A subcutaneous neoplasm (syringoma) occurred in both groups, the incidence being roughly the same (4 experimentals, 3 controls). The overall mortality was elevated in the experimental group (5 animals, as compared with 2 in the controls), but no specific cause of death could be established.

On autopsy no unequivocal macroscopical or microscopical changes were seen. The relative organ weights (heart, spleen, liver, kidneys, lungs, testes, ovaries) did not differ significantly between groups.

D. *Chickens*

1. *Single dose*

Three groups of 3, 6 and 2 New Hampshire chickens, respectively, were given 2,4-D amine as a single oral dose of 100, 200 and 300 mg/kg, respectively.

Clinical and gross pathological findings were negative, except for gastritis in one animal on the highest dose. Maximum plasma levels of 2,4-D averaged 90, 130 and 250 μg/ml, respectively, in the three groups. In all animals the level had decreased to 15 μg/ml or less at 24 hours.

2. *Short-term experiment*

Three chickens (New Hampshire) were given 2,4-D amine as repeated oral doses of 300 mg/kg/day. One animal died on the fifth day; renal and visceral gout were found on autopsy. In the remaining animals, killed after 12 and 24 days, respectively, no specific gross pathological changes were seen, except for slight kidney enlargement. An enhanced elimination rate of 2,4-D was noted, however, the average plasma value level having dropped to 20 μg/ml already at 3 hours.

3. *Long-term experiments*

a) *Chickens.* Six chickens (Leghorn) were fed 2,4-D amine at a level of 500 p.p.m. in standard feed.

One animal died after 5 months. On autopsy hypoplasia (possibly congenital) of the right kidney and hyperplasia of the left kidney were seen. The cause of death was renal gout. The other animals exhibited no distinct clinical symptoms. They were killed after 1, 2, 9 and 18 (2 animals) months, respectively. Autopsy findings were respectively: acute, waxy muscular degeneration; stomach ulcer and lymphocytic peribronchitis; generalized anaemia; no specific lesions (the last 2 animals).

b) *Day-old chicks.* Day-old broiler chicks were randomly distributed between one experimental (29 animals) and one control group (25 animals). For the first three weeks the animals were kept in rearing batteries, then for one month in groups of 3 in cages and thereafter in individual cages. From the third day on the experimental group received 2,4-D amine at 1000 p.p.m. in the drinking water.

Weight gain, age at sexual maturity and onset of egg production did not differ significantly between experimental animals and controls. The number, and possibly the weight, of eggs, however, was significantly reduced in the experimental group. In order to eliminate variation due to different times of death, only the first two months of laying were compared (Table 3).

Table 3. Average number and weight of eggs, during the first two months of laying, in hens receiving 2,4-D amine (1000 p.p.m.) in the drinking water.

Group	Number per hen (m ± s)	Weight, g (m ± s)
Experimental (n = 6)	22 ± 4.7	41 ± 3.1
Control (n = 7)	33 ± 1.0	45 ± 2.3

Part of the ingested 2,4-D was excreted with the eggs, levels of 1—2 μg/g being found in the yolk and traces in the white.

The total number of animals dead or killed because of disease did not differ between groups (experimentals 8 and 3, respectively; controls 5 and 7, respectively).

The causes of death or killing were as follows.

Experimental group: coccidiosis (2 animals), enteritis of unknown origin (1), leukosis (1), lymphomatosis (1), generalized anaemia and pulmonary oedema (1), inanition of unknown origin (2), traumatic lesions (1), not confirmed (2).

Control group: coccidiosis (2), enteritis of unknown origin (3), coprostasis (1), leukosis (2), lymphomatosis (1), perosis (2), inanition of unknown origin (1).

Surviving animals were killed and autopsied at intervals between 2 and 18 months, an equal number of controls always being examined in parallel with the experimental animals.

The main macroscopical finding was consistent enlargement of the kidneys in experimental animals, observable in dead, as well as in killed animals; the kidneys were pale and had a firm consistency (Fig. 6). The relative kidney weight (per cent of body weight, m ± s) was 1.80 ± 0.13, as compared to 0.66 ± 0.05 for the controls. The difference is statistically highly significant ($t = 11.2$). The other organ weights did not differ significantly between groups.

On microscopical examination the kidney enlargement could be attributed to epithelial hypertrophy and hyperplasia of varying tubular sections with a concomitant slight to moderate dilatation, the changes being most conspicuous in the proximal convoluted tubules (Figs. 7 and 8). The collecting tubules were slightly to moderately dilated (Figs. 9 and 10). In addition, the glomerular and tubular basement membranes appeared unusually distinct, particularly on PAS staining, and the dense glomerular tufts showed an enhanced stainability and also contained a reduced

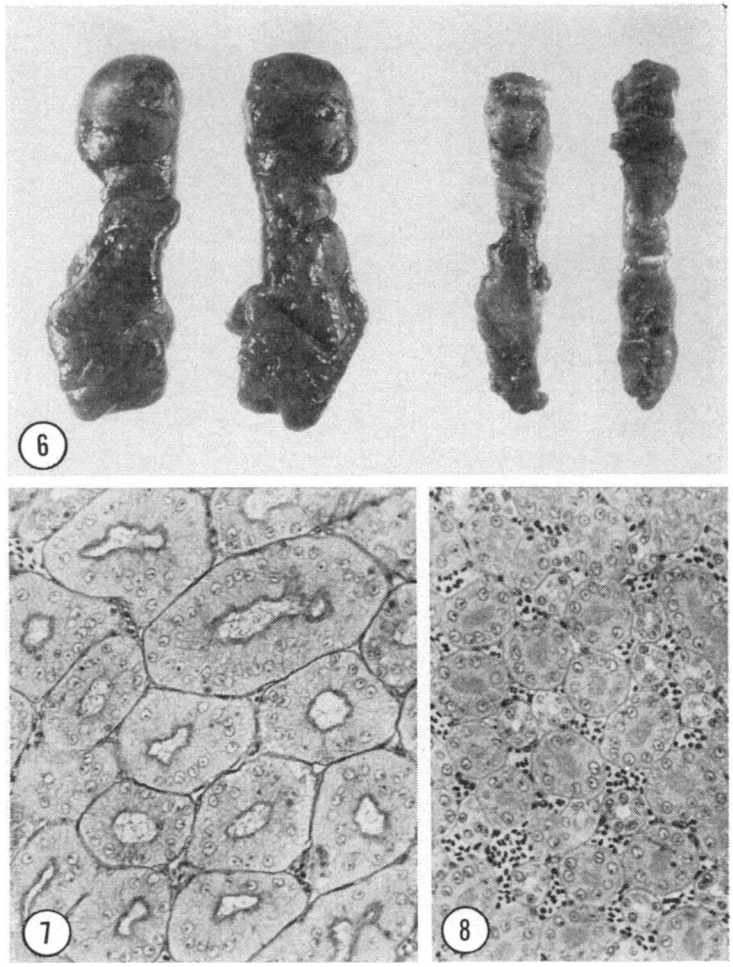


Figure 6. Hypertrophic kidneys (left) of chicks given 2,4-D (1000 p.p.m.) in drinking water for 5 months, and kidneys of normal chicks of the same age (right).

Figure 7. Section of hypertrophic kidney of Fig. 6. Tubular hypertrophy. PAS, 240 ×.

Figure 8. Section of normal kidney of Fig. 7.

number of cells (Figs. 11 and 12). In preliminary experiments with a higher dose level (2000 p.p.m. in the feed) a massive dilatation of the tubules as well as of the collecting ducts, with partly desquamated epithelium was notable (Figs. 13 and 14). Excessive glomerular lesions were also seen in those cases, mainly

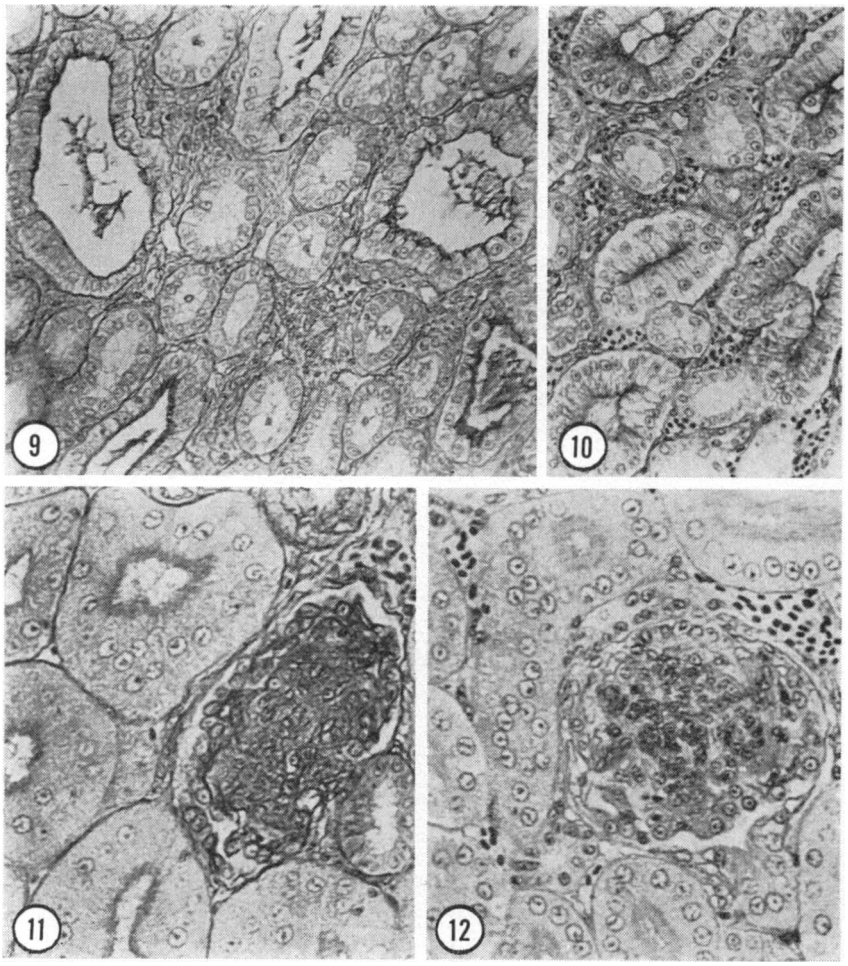
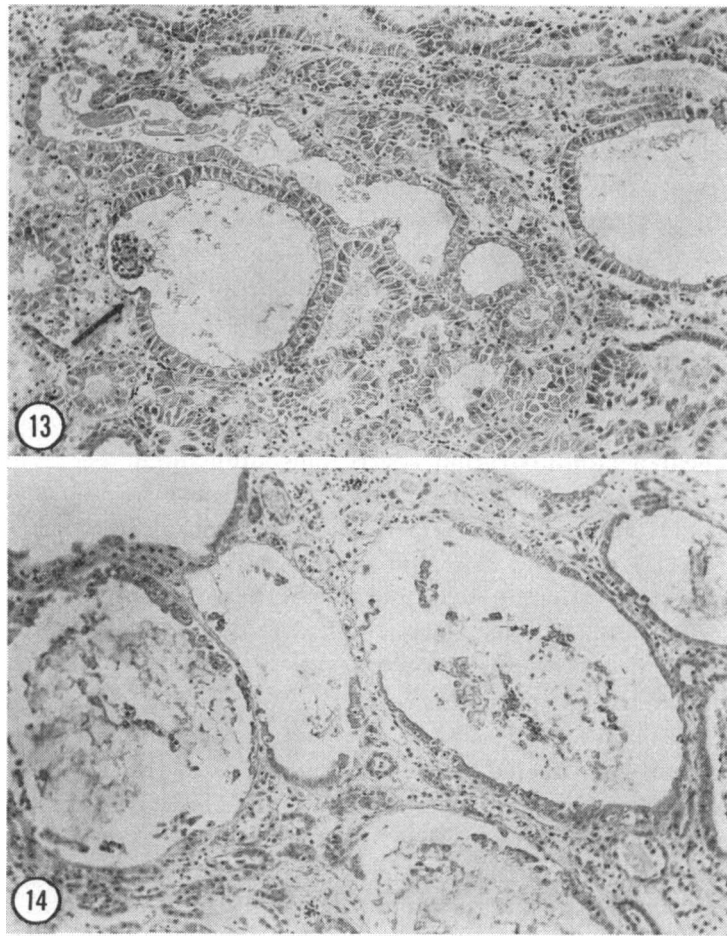


Figure 9. Kidney of chick given 2,4-D (1000 p.p.m.) in drinking water for 5 months. Moderately dilated collecting tubules. PAS, 400 ×.

Figure 10. Kidney of normal chick.

Figure 11. Kidney of chick given 2,4-D (1000 p.p.m.) in drinking water for 5 months. Dense and distinctly PAS-positive glomerular tufts and clearly visible tubular and glomerular basement membranes. Slightly dilated tubules with epithelial hypertrophy. PAS, 400 ×.

Figure 12. Kidney of normal chick.



Figures 13 and 14. Kidney of chick given 2,4-D (2000 p.p.m.) in feed for 2 months, cortex (Fig. 13) and medulla (Fig. 14). Extreme dilatation of Bowman's capsule (arrow, Fig. 13), tubules and collecting ducts. Haematoxylin and eosin, 120 ×.

as epithelial hypertrophy and hyperplasia of Bowman's capsule with a consequent dilatation of the capsular cavity. In such cases, the appearance of the kidney was reminiscent of a cystic adenoma.

It should be remarked that no signs of renal gout were seen in the experimental animals.

DISCUSSION

Acute and subacute toxicity. As indicated by the preliminary acute-toxicity experiments, 2,4-D might cause definite but reversible toxic effects in *calves* after a single oral dose of 200 mg per kg body weight, slight effects being noted also at lower doses. The result, as well as the main clinical symptoms, are consistent with the toxicity data reported by *Rowe & Hymas* (1954) and by *Palmer & Radeleff* (1964). The occurrence of dysphagia in cattle in this connection, however, does not seem to have been reported previously, although it was observed in 2,4-D-poisoning in dogs by *Drill & Hiratzka* (1953).

The minimum acutely toxic dose of 2,4-D in *pigs* was about 100 mg/kg orally and the fatal dose of the order of 500 mg/kg. On repeated administration severe poisoning might occur after less than 10 doses of 50 mg/kg and after 3 doses of 100 mg/kg (Table 1), indicating a relatively high susceptibility in this species. This seems to be in line with results of a previous study indicating a relatively low elimination rate for phenoxy acids in pigs *(Erne* 1966 b), the plasma half-life of 2,4-D being around 12 hours. Moreover, the plasma levels of 2,4-D in animals developing signs of poisoning on repeated administration were invariably high and persistent. As a contrast, in animals remaining clinically unaffected by the repeated exposure, the elimination rate was seen to increase after some time. A comparison of plasma levels and clinical effects seem to suggest a correlation between the plasma level and the onset and duration of symptoms. Thus, definite signs of poisoning should not be likely to appear in pigs at plasma levels below a threshold value of about 200—300 µg 2,4-D/ml. Apparently, conditions are similar in calves; the rat and chicken experiments do not allow any inferences in this respect because of the absence of clinical signs. The main symptoms in acute or subacute poisoning in pigs were anorexia and transient diarrhoea and, in severe cases, vomiting, muscular weakness and general depression. Apart from signs of gastrointestinal irritation the histopathological picture was dominated by lesions in the lungs or the bronchial lymph glands, or both. Inasmuch as these changes were seen independently of the mode of administration, they cannot be a result only of aspiration in connection with intubation. Furthermore, virus pneumonia (SEP) seems less probable as an aetiological factor, since infections of this type did not at all occur in the animal quarters at the time

of the experiments. In experimental poisoning with phenoxy herbicides in other species, manifestations from the respiratory organs have been reported frequently. The pathogenesis of these changes, however, is not known.

In *rats* a single oral dose of 2,4-D of 100 mg/kg caused no visible effects. The experiment was performed mainly in order to obtain distribution data (*cf. Erne* 1966 b). *Chickens,* in agreement with previous results (*Rowe & Hymas*), exhibited a comparatively high tolerance for 2,4-D, single doses of up to 300 mg/kg, as well as daily doses of the same order given for 2—3 weeks, apparently being tolerated without adverse effects. Gross and microscopical changes were not detected in the short-term experiments with rats and chickens.

In accordance with previous work (*Hill & Carlisle* 1947; *Rowe & Hymas*), our experiments did not reveal any noticeable difference in acute toxicity between a commercial formulation of 2,4-D and the pure chemical. A previous study showed the two types of preparation to be similar in respect to plasma levels attained (*Erne* 1966 b). The butyl ester of 2,4-D, when given orally, produced only low plasma levels of 2,4-D and also had a low acute toxicity, both effects presumably being attributable to the poor water solubility and the consequent low absorption rate of this compound. As regards toxicity, as well as plasma levels, there seemed to be no essential difference between 2,4-D and 2,4,5-T.

Long-term effects. Pigs. Beside symptoms similar to, although less severe than, those seen in the short-term experiments, depression of growth and persistent anaemia were conspicuous after feeding 2,4-D to pigs over extended periods. It is true that the experimental feed appeared unpalatable to the animals, but since the ration was completely consumed each day, the growth depression should probably be regarded as a general toxicity effect rather than as a result of malnutrition. The average daily dose of 2,4-D estimated from the feed intake was approximately 25—50 mg/kg body weight.

Muscular degeneration was not present, and therefore the slightly elevated GOT values, together with slightly reduced plasma albumin levels and morphological changes in the liver cells, suggest some hepatotoxic action in the pigs. Changes of the type seen, parenchymatous degeneration and nuclear variability of hepatic cells, are generally regarded as signs of an adap-

tation after a repeated deleterious action (*Beneke & Simon* 1961). Signs of renal dysfunction were not conclusive; despite the fact that albuminuria was common, the morphological lesions of the kidneys were only slight.

In view of the often reported occurrence of locomotory disturbances in phenoxy herbicide poisoning in other species, the ungular and the spinal changes seen in some animals should attract interest. The significance of these changes, however, cannot be assessed until their relationship with the intake of phenoxy acid has been substantiated.

The experiment with the pregnant sow allowed the effect of continuously ingested 2,4-D to be followed over two generations. Although the sow did not display any effects which could be associated with the intake of 2,4-D, the offspring was clearly affected. The major manifestations in the newborn piglets were underdevelopment, apathy and a high mortality rate, 10 out of 15 dying within 24 hours. No malformations were seen. In the survivors growth depression and anaemia were evident. Even if an old-age fertility impairment may have modified the result of this experiment, the effects on the newborn piglets were so pronounced that an interference of the phenoxy acid with reproduction cannot be excluded. Compatible with this view is also the result of chemical analyses, indicating the presence of relatively high levels of 2,4-D in the tissues of the newborn piglets, and hence the ready passage of the phenoxy acid across the placental barrier (*Erne* 1966 b).

Rats. As in the pig experiments, the effect of continued administration of 2,4-D was followed over two generations. At a dosage, calculated on basis of the water consumption to correspond to roughly 50—100 mg/kg/day, the mothers remained clinically normal throughout the experiment, while the young rats showed reduced feed and water intake and retarded growth as compared to controls. On autopsy no definite abnormalities were observed in any generation. These comparatively mild effects may be related to the high excretory capacity for phenoxy acids demonstrated in rats (*Erne* 1966 b), the plasma half-life of 2,4-D being as low as 3 hours. Previous workers have observed growth depression and tissue changes after prolonged daily administration of 100 mg/kg (*Rowe & Hymas*; *Thomssen* 1958), and also after feeding 1000 p.p.m. in the diet for several months (*Rowe & Hymas*). Clinical and morphological changes were seen

by *Schillinger* (1960) at 500 mg/kg given daily for an extended period; a reversible catalase inhibition was observed at 50 mg/kg when given daily for one year.

Chickens. In the long-term experiments with chickens, 500 p.p.m. of 2,4-D in the feed and 1000 p.p.m. in the drinking water caused morphological changes although the clinical effects were slight. The average daily dose of 2,4-D could be roughly estimated to between 50 and 100 mg/kg in both instances. The results are compatible with recorded experience of other workers. *Bjorn & Northen* (1948) found 12 doses of 280 mg 2,4-D/kg, given during 4 weeks, to depress the growth of chickens, while 12 doses of 28 mg/kg did not; the single lethal dose was between 380 and 765 mg/kg. In the experiments of *Rowe & Hymas,* 1000 p.p.m. of 2,4-D given in the feed for 7 days was tolerated without clinical effects, whereas 3000 p.p.m. caused a reduced feed intake and retarded growth. *Andersson et al.* (1962) observed no effect on the growth rate of chicks after feeding either 2,4-D or 2,4,5-T or a mixture (all in the ester form) at the rates of 192, 250 and 510 p.p.m., respectively, for 8 weeks. At the rates of 1920, 2500 and 5100 p.p.m., however, conspicuous growth inhibition and a high mortality were seen.

The most prominent toxic manifestations in our experiments were, except for the reduced egg production in laying hens, the renal hypertrophy in chicks.

A reduced egg production is not an uncommon response in hens, which may be elicited by toxic agents as well as by various nutritional and environmental factors. The renal lesions, on the other hand, do not appear to have been described previously. The fact that only the avian species responded in this way may, at least partly, have a physiological background; the avian kidney recieves, in addition to arterial blood, also venous blood via a renal portal system, and as a result substances from certain parts of the intestinal tract may be transferred directly to the kidney. Physiological evidence suggests that most of the afferent blood to the tubules comes from this portal system (*Sturkie* 1965). However, some additional factor obviously is involved, since no appreciable renal response was observed, when adult chickens were used as experimental animals. The characteristic, almost adenomatous proliferations did develop only when newly-hatched chicks were repeatedly exposed to the phenoxy acid. In experiments with leukaemia virus, *Carr* (1956) found that renal adeno-

carcinoma could be induced in chicks only when exposed during the first two weeks after hatching. As pointed out by *Siller* (1962), the reactivity in that case may be associated with the abundance of residual embryonic tissue in very young birds. Possibly the age-dependent susceptibility to chemically-induced lesions may be interpreted similarly. The epithelial proliferations of Bowman's capsule observed in extreme cases in our material pathogenetically may be related to a physiological alteration of renal tissue frequently seen in young chicks, *viz.* cuboidal metaplasia of the flattened squamous capsular epithelium, preferentially taking place at 2—3 weeks and at 5 weeks of age (*Siller*). Since the phenoxy acids are being enriched in renal tissue during excretion they should have an, at least theoretical, opportunity of interfering with developmental processes like this one.

In concluding, our results seem to indicate the chronic toxicity of 2,4-D for the species studied to be only moderate. Apart from the appreciable nephrotoxicity demonstrated in young chicks, the long-term effects were signs of a general toxicity. The high mortality in the newborn piglets and the reduced egg production in chickens merit attention, however, as being possible signs of an interference with the reproductive cycle.

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SUMMARY

The main purpose of the present work was to study the long-term effects of 2,4-dichlorophenoxyacetic acid (2,4-D) in swine, rats and chickens.

In preliminary short-term experiments with calves and pigs, definite although reversible toxic effects were seen after single doses of 200 and 100 mg/kg, respectively. Rats and chickens seemed to tolerate 100 and 300 mg/kg, respectively, without ill-effects. On repeated administration daily doses of 50 mg/kg could be toxic to pigs, whereas chickens tolerated 300 mg/kg/day for several weeks without visible effects. Symptoms of acute poisoning in calves were dysphagia, anorexia, tympanites and muscular weakness. Anorexia was apparent also in acutely or subacutely poisoned pigs together with locomotory disturbances, transient diarrhoea and, in severe cases, vomiting, muscular weakness and general depression. In all animals showing symptoms of poisoning a reduced disappearance rate of 2,4-D from plasma was apparent. On autopsy the pigs showed signs of

gastro-intestinal irritation and pneumonia and renal degeneration. In the rats and chickens no gross pathological changes were seen.

In the long-term studies 5 young pigs were fed 2,4-D (500 p.p.m.) for up to 12 months. Main clinical signs were growth depression, locomotory disturbances, anaemia and albuminuria. Morphological changes included moderate hepatic and renal degeneration. In another experiment 2,4-D was fed to a pregnant sow throughout the gestation period and for 6 further weeks. The sow exhibited no characteristic signs, and on autopsy no changes attributable to 2,4-D were noted. The newborn piglets, however, were underdeveloped and apathetic. Ten out of 15 died within 24 hours. On continued feeding of 2,4-D to the survivors until 7—8 months of age the main effects were a marked growth depression, persistent anaemia and moderate degenerative changes of liver and kidneys.

Pregnant rats were given 2,4-D (1000 p.p.m.) in the drinking water during the gestation and further for up to 10 months. The administration of 2,4-D was continued to the second generation rats for up to 2 years. Except for a retarded growth and an increased mortality in the second generation no unequivocal clinical or morphological changes were seen.

In chickens continued administration of 2,4-D (500 p.p.m. in the feed or 1000 p.p.m. in the drinking water) caused a reduced egg-production and pronounced kidney enlargement due to epithelial proliferations, this latter lesion appearing only when very young chicks were used as experimental animals.

The experimental results indicate the chronic toxicity of 2,4-D for the species examined to be moderate. Apart from the nephrotoxicity demonstrated in chicks the long-term effects were non-specific. Of particular interest, however, are the high mortality in the newborn piglets and the reduced egg production in the chickens as indications of a possible interference with reproduction.

ZUSAMMENFASSUNG

Toxikologische Studien über Phenoxyessigsäure-herbiziden.

Da verhältnismässig häufig Schadenfälle mit Verdacht auf akute oder chronische Phenoxy-herbizidvergiftung bei Haustieren und beim Wild vorgekommen sind, wurden hier einige Versuche ausgeführt, um den Effekt anhaltender Zufuhr von 2,4-Dichlorphenoxyessigsäure (2,4-D) an Schweine, Ratten und Hühner zu studieren.

In vorläufigen Versuchen mit Kälbern und Schweinen wurden bestimmte, aber vorübergehende toxische Effekte durch 200 bzw. 100 mg/kg hervorgerufen. Ratten und Hühner vertrugen 100 bzw. 300 mg/kg ohne klinische Symptome. In wiederholter Dosis waren 50 mg/kg toxisch für Schweine. Hühner vertrugen 300 mg/kg täglich während 2—3 Wochen ohne schädliche Wirkung. Symptome der akuten Vergiftung waren bei Kälbern Schluckbeschwerden, Appetitlosigkeit, Meteorismus und Muskelschwäche. Inappetenz trat auch bei Schweinen zusammen mit Bewegungsstörungen, Diarrhöe und, in

schwierigen Fällen, Erbrechen, Muskelschwäche und Depression auf. Bei allen Tieren, die klinische Vergiftungssymptome aufwiesen, wurde eine herabgesetzte Eliminierungsgeschwindigkeit von 2,4-D aus dem Plasma nachgewiesen. Sektionsbefunde waren bei den Schweinen Magen- und Darmreizung sowie Lungen- und Nierendegeneration. Die Ratten und Hühner waren makroskopisch negativ.

In den chronischen Toxizitätsversuchen wurden jungen Schweinen 2,4-D (500 p.p.m.) im Futter während der Zeit von 2—12 Monaten verabreicht. Klinisch wurden hauptsächlich Wachstumshemmung, Bewegungsstörungen und Anämie sowie Albuminurie wahrgenommen. Morphologisch wurde bei geschlachteten Tieren geringe Leber- und Nierendegeneration beobachtet. Ausserdem wurde 2,4-D einer trächtigen Sau während der Trächtigkeit und für 6 weitere Wochen im Futter verabreicht. Die Geburt brachte 15 Ferkel, die unterentwickelt und apathisch waren, und von denen 10 innerhalb 24 Stunden starben. Bei fortgesetzter Zufuhr von 2,4-D im Futter an die Überlebenden während der Zeit von 7—8 Monaten waren dauernde Anämie und Wachstumshemmung die auffälligsten Effekte. Geringe Leber- und Nierendegeneration wurde auch beobachtet. Die Sau wies keine bemerkenswerten Symptome auf und bei der Sektion auch keine charakteristischen Veränderungen.

Trächtigen Ratten wurden 2,4-D (1000 p.p.m.) im Trinkwasser während der Trächtigkeit und ferner 10 Monate lang zugeführt. Die Verabreichung von 2,4-D wurde von der zweiten Generation 2 Jahre lang fortgesetzt. Ausser Wachstumshemmung und Mortalitätserhöhung in der zweiten Generation liessen sich keine eindeutigen klinischen oder morphologischen Effekte feststellen.

An Hühnern, denen 2,4-D im Futter (500 p.p.m.) oder Trinkwasser (1000 p.p.m.) dauernd verabfolgt wurden, war eine verminderte Eiproduktion wahrnehmbar. Ferner war Nierenvergrösserung zufolge charakteristischer Epithelproliferation ein auffälliger Befund, eine Erscheinung, die allerdings nur hervorgerufen werden konnte, wenn sehr junge Küken als Versuchstiere verwendet wurden.

Die Versuchsergebnisse deuten auf eine mässige chronische Toxizität von 2,4-D bei den untersuchten Tierarten hin. Von den bei den Küken beobachteten Nierenschädigungen abgesehen, waren die Effekte hauptsächlich unspezifisch. Von Interesse sind doch die Effekte an den neugeborenen Ferkel und der Eirückgang bei den Hühnern, als Symptome eines möglichen reproduktionsstörenden Effekts der Phenoxyverbindung.

SAMMANFATTNING

Toxikologiska undersökningar över fenoxiättiksyreherbicider.

Huvudsyftet med här beskrivna försök var att studera långtidseffekten av 2,4-diklorfenoxiättiksyra (2,4-D) på svin, råttor och höns.

I inledande försök med kalvar och grisar iakttogs en definitiv men reversibel giftverkan efter orala engångsdoser av 200 resp. 100 mg/kg. Råttor och höns tolererade engångsdoser av 100 resp. 300

mg/kg utan synbar påverkan. Vid upprepad tillförsel iakttogs en tydlig skadeverkan på grisar vid en daglig dos av 50 mg/kg, medan höns tålde 300 mg/kg/dag under flera veckor utan kliniska symtom. Akuta förgiftningssymtom var hos kalv sväljningsbesvär, inappetens, trumsjuka och muskelsvaghet. Även hos grisar sågs vid akut eller subakut förgiftning inappetens och dessutom rörelsestörningar, diarré och, i svårare fall, kräkning, stark muskelsvaghet och allmän depression. Hos alle djur med kliniska förgiftningssymtom iakttogs en nedsatt elimineringshastighet från plasma av 2,4-D. Vid obduktion av grisarna påvisades tecken på gastro-intestinal irritation och pneumoni samt degenerativa njurförändringar. Hos råttorna och hönsen iakttogs inga makroskopiska förändringar.

I långtidsförsök med grisar tillfördes djuren 2,4-D i fodret i en halt av 500 p.p.m. under upp till 12 månader. Härvid observerades huvudsakligen tillväxthämning, rörelsestörningar, anemi och albuminuri samt lindriga degenerativa förändringar i lever och njurar. I ett annat försök gavs 2,4-D i fodret till en dräktig sugga under hela dräktighetsperioden och under ytterligare 6 veckor. Suggan visade inga karakteristiska symtom och vid slakt ej heller patolog-anatomiska förändringar, som kunde sättas i samband med 2,4-D-tillförseln. Under en utdragen förlossning föddes 15, underviktiga och apatiska, smågrisar, varav 10 dog inom 24 timmar. Vid fortsatt tillförsel av 2,4-D till de överlevande, till 7—8 månaders ålder, iakttogs främst en stark tillväxthämning och bestående anemi och därjämte lindrig degeneration av lever och njurar.

Dräktiga råttor tillfördes 2,4-D (1000 p.p.m.) i dricksvattnet under dräktighetsperioden och under ytterligare 10 månader. Tillförseln av 2,4-D fortsattes till ungarna under upp till 2 år. Utöver tillväxthämning och ökad mortalitet hos andra generationen kunde inga entydiga kliniska eller morfologiska förändringar iakttas hos försöksdjuren.

Vid kontinuerlig tillförsel till höns av 2,4-D i fodret (500 p.p.m.) eller i dricksvattnet (1000 p.p.m.) iakttogs en nedsatt äggproduktion och dessutom markerade njurförändringar med förstoring till följd av epitelproliferation, vilka senare skador endast kunde framkallas, när mycket unga kycklingar användes som försöksdjur.

Erhållna försöksresultat antyder en måttlig kronisk toxicitet hos 2,4-D för undersökta arter. Bortsett från njurförändringarna hos kycklingar var iakttagna effekter ej specifika. Särskild uppmärksamhet förtjänar dock den höga spädgrismortaliteten och den minskade äggproduktionen hos höns, såsom tecken på en potentiell reproduktionsstörande effekt hos fenoxisyror.

(Received June 29, 1966).