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# Toxicity of 4-Chloro-O-Cresol to Rat: I. Light Microscopy and Chemical Observations

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4-chloro-o-cresol was first identified as the metabolite of 2-methyl-4-chlorophenoxyacetic acid (MCPA), a widely used herbicide by GAUNT and EVANS (1961), later by BOLLAG et al. (1967) and it has been shown to be a metabolite of other phenoxyacetic acid herbicides, too (BJERKE et al. 1972). It has been shown to act as an acid catalyst in oxidative phosphorylation (YAGUZHINSKII et al. 1971) and affect artificial phospholipid membranes (MARKIN et al. 1971). The toxicity of the compound has not been studied so far.

In this work the toxicity of 4-chloro-o-cresol was studied in male rats in order to relate the histopathological changes in the tissues to the residue levels in the same tissues. A new TLC-method was used to separate the cresol from fat and chlorinated catechols. Glass capillary GLC was used in the quantitative determination of the compound.

## MATERIALS AND METHODS

The rats used were 2-3 months old male Wistar rats, the average weight of which was 380 g in the acute experiments and 290 g in the subchronic experiment.

The cresol used was purified to 100% purity from 4-chloro-o-cresol (Fluka, purum) in the Department of Chemistry in the University of Jyväskylä and the purity was checked by IR-, NMR- and mass-spectrometry. The compound was dissolved in olive oil and applied by a 5 ml syringe equipped with a steel canyl directly in the stomach.

## EXPERIMENTAL

Acute experiments: The $LD_{50}$-value was determined by the method of WEIL (1952) both by intraperitoneal and oral administration. Only the rats of the latter experiment were taken for analyses. In the acute experiment in which the cresol was applied orally following concentrations were used: Group I 1000 mg/kg, Group II 1100 mg/kg, Group III 1200 mg/kg and Group IV which served as a control. 10 animals were taken in each group. No animals died from the first group in 24 h, one animal died from Group II and nine from Group IV.

After 24 h the animals were killed and tissue samples for light microscopy were taken from liver, kidney, spleen and muscle (musculus vastus lateralis) and fixated in 10% buffered formalin and stained with haematoxylin eosin. For the chemical analysis

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samples were taken from the same tissues, wrapped in aluminium foil and deep frozen in -25°C until analyzed.

Subchronic experiments: In the subchronic experiment 10 animals were taken in each group and the following concentrations of cresol were applied: Group I 100 mg/kg, Group II 250 mg/kg, Group III 500 mg/kg. 10 animals were taken for the control group.

The cresol was injected daily during four weeks and the food consumption was checked. The animals were also weighed daily. Histopathological samples were taken as in the acute experiment from liver, kidney, spleen, skeletal muscle, stomach, small intestine and large intestine. For the chemical analyses samples were taken from liver, kidney, spleen and muscle.

Extraction of residues: The frozen sample was melted and homogenized in Sorvall Omnimixer with anhydrous $Na_2SO_4$, which was used 3 g/g of wet tissue. The sample was dried for 24 h at room temperature. The extraction was carried out by shaking the homogenate 3 x 0.5 h with chloroform-diethylether 1:1 (v/v) made acidic with fuming HCl (pH < 3) which was applied 30 ml for 1-2 g of wet tissue and 50 ml for 2-5 g, respectively. The shaking occurred in a Griffin flask-shaker. The extracts were filtered through filter paper (Whatman No. 1) and the combined extracts were evaporated in Büchi evaporator. The residue was dissolved in 2-3 ml of ether and transferred quantitatively to 10-ml volumetric flasks and filled to mark. 1 ml of the solution was evaporated carefully with nitrogen (99.999% purity) and applied for TLC.

Clean up: TLC plates 24 x 24 cm made of Kieselgel G nach Stahl (layer thickness 1 mm) were used for qualitative and quantitative separation of cresol from other compounds.

The activated plate was divided in 5 sections by upright lines by pencil of which 4 were used for samples and one for standard. The extraction residue was applied by Carlsberg micropipette in one spot (1 cm from the lower end), maximum fat load was 30 mg and in the last section 10 µl pure 4-chloro-o-cresol (10 µg/ml) was applied and the plate was developed in redistilled dichloromethane.

Several solvent systems were tried but dichloromethane was chosen because it was possible to separate also MCPA, chlorinated cresols and catechols from fat by one chromatographing only.

The plate was dried at room temperature and wrapped tightly in aluminium foil with the exception of the standard. The plate was sprayed with 2% 2,6-dichlorochinonchlorimid in benzene which made cresol visible (bright blue). The $R_f$-value for cresol in this method was 0.48.

Quantitative analysis: According to the visible spot the cresol was then scraped by a razor blade in a glass-wool stoppered tube (10 x 1 cm) with a pipet end and the fraction was eluted by ether in 1-ml volumetric tube. The recovery of this method for the concentration of 10 and 20 µg 4-chloro-o-cresol in 1 ml was 83.7% ($\pm$13.4). The solution was then chromatographed by

GLC with glass capillary column. The equipment used was Carlo Erba Model Fractovap equipped with FID-detector and a grop-type split-less injection system. The column used was 15 m FFAP, inside diameter 0.35 mm. Standards treated by the method described were chromatographed daily in three concentrations and the quantitative determination occurred by comparing the peak heights. In the lower tissue concentrations (100 mg/kg) each extract was first developed by TLC and if no visible spot was obtained the GLC was not carried out.

Histopathological studies: Acute. The $LD_{50}$-value when cresol was applied intraperitoneally was 794 mg/kg and 1194 mg/kg when oral administration was used. The histopathological studies showed that at the dose levels 1000 and 1100 mg/kg muscle, spleen and liver were normal. In the kidney inflammatory mononuclear infiltration was seen in many glomeruli. In some samples almost every glomerulus was affected. Inflammatory infiltrations were also seen in other parts of the kidney, mostly around distal tubules. At the dose level of 1200 mg/kg the changes were similar to the previous groups, except that histopathological alterations were also seen in the liver and spleen. In the liver numerous pycnotic nuclei and hydropic degeneration of cytoplasm were observed. In the pericentral areas inflammatory infiltrations were also seen. In the spleen the reaction centers were unusually large.

Subchronic. During the experiment the food consumption and weight development in all test groups were similar to the controls. At the dose level of 100 mg/kg all other tissues were normal except the small intestine. In the mucosa necrotic areas were seen and there were extensive mononuclear infiltration in the underlying tissues. Similar but smaller necrotic lesions were also seen in the glomeruli, as well as in the interstitium between the proximal tubules. The spleen and muscle tissues were normal. In addition to histopathological studies blood analyses were carried out. These results are given in Table 1. It is seen that no difference is found in haemoglobin, haematocrite or in total eosinophilic count or lymphocyte count. The leucocytes on the other hand decrease with larger doses and with the dose of 500 mg/kg there is clear-cut leucopenia.

Chemical analysis: Acute. The results expressed as mg/kg residue in the tissues are presented in Table 2. The results show that at the dose levels of 1000 and 1100 mg/kg the highest concentrations, about 20 mg/kg, were observed in the kidney. At the dose level of 1200 mg/kg which was above the $LD_{50}$-level a very high concentration, about 100 mg/kg was observed in the liver and kidney.

Subchronic. The concentrations of 4-chloro-o-cresol in different tissues are presented in Table 2. At the dose level of 100 mg/kg only traces of the residue were found. Blue spots were developed by TLC indicating the presence of the compound but the concentrations were under the limit of the detection by GLC. At the dose levels of 250 and 500 mg/kg the highest concentrations

TABLE 1

Composition of blood in the subchronic experiment.

| Dose mg/kg | Hg g/l | H kr % | $L_2$ $mm^2$ | E $10^6$ mm | Differential leucocytes: Polymorphonuclear leucocytes | L-monocytes | L-lymfocytes |
|---|---|---|---|---|---|---|---|
| 0 | 162 | 48 | 6100 | 7.76 | 8.5 | 2.8 | 87.0 |
| 100 | 160 | 49 | 5200 | 8.30 | 6.3 | 1.9 | 91.2 |
| 250 | 160 | 48 | 5900 | 8.08 | 5.8 | 2.7 | 90.9 |
| 500 | 153[x] | 48 | 4100 | 8.13 | 10.5 | 2.2 | 86.9 |

[x]The test by t-test showed significant difference (risk 1% compared with the control)

TABLE 2

The concentration of 4-chloro-o-cresol mg/kg wet weight in different tissues after acute and subchronic exposure of the compound.

| Dose (mg/kg) | Liver | Kidney | Spleen | Muscle |
|---|---|---|---|---|
| Acute | | | | |
| 1000 | 11.64 | 22.17 | 8.39 | x |
| $s_d$ | 3.57 | 2.13 | 3.39 | |
| 1100 | 11.86 | 20.72 | 17.33 | x |
| $s_d$ | 2.93 | 7.75 | | |
| 1200 | 96.12 | 93.37 | 103.32 | 37.01 |
| $s_d$ | 24.34 | 20.79 | 18.30 | 14.43 |
| Subchronic | | | | |
| 100 | tr | tr | tr | - |
| $s_d$ | | | | |
| 250 | 0.26 | 0.82 | 0.97 | 0.24 |
| $s_d$ | 0.09 | 0.20 | 0.13 | 0.13 |
| 500 | 0.51 | 1.20 | 2.81 | 0.27 |
| $s_d$ | 0.14 | 0.29 | 0.53 | 0.06 |

x not analyzed chemically
tr traces

0.969 and 3.221 mg/kg were observed in the spleen and following was the kidney in both groups. The lowest concentrations were observed in the muscle.

## DISCUSSION

MCPA is the most widely used herbicide in the Nordic countries. The metabolism and toxicology of MCPA has been studied

only little and not at all in humans. GAUNT and EVANS (1961) showed that the first metabolite of MCPA in soil was 4-chloro-o-cresol. The toxicity of 4-chloro-o-cresol has not been studied so far. One commercial product of MCPA has been studied in our laboratory and it contained 4-chloro-o-cresol as an impurity approximately 4%.

In our study when 4-chloro-o-cresol was applied directly into the stomach in the acute experiment, the highest concentrations were found in the kidney and spleen. Also in the subchronic experiment the highest concentrations were observed in the same organs. The lowest concentrations in both the experiments were found in the skeletal muscle.

The histopathological studies showed that in the acute experiment at the dose levels of 1000 and 1100 mg/kg inflammatory infiltrations were common in the kidney and at the dose level of 1200 mg/kg morphological changes were observed also in the liver and spleen. In the liver the nuclei were often pycnotic and necrotic scattered cells were found. In the spleen the reaction centers were enlarged.

In the subchronic experiment at the lower dose level, 100 mg/kg, all the other tissues were normal except the mucosa of the small intestine in which extensive necrosis was observed. At the dose levels of 250 and 500 mg/kg changes were also observed in the liver and in the kidney in addition to small intestine and large intestine. As a rule the greatest changes in the histopathology were seen in the organs in which the concentrations were highest.

In the subchronic experiment the chemical concentrations in the organs were relatively low. This may due to two facts, firstly, that the cresol is metabolized further and secondly, that it has been poorly absorbed from the stomach and small intestine. The latter possibility is supported by the fact that extensive histological changes were found in the mucosa of the small intestine.

The blood analyses showed that with larger doses 4-chloro-o-cresol induces slight leukopenia, whereas otherwise the blood cells are only little affected.

When the results of the present study are compared with those obtained in the acute and subchronic experiments with MCPA (HATTULA et al. 1977) following observations can be made.

In the acute experiment with MCPA the greatest changes were observed in the liver and spleen. In the liver sinusoids were hyperemic and in the parenchyma necrotic areas were found. The necrotic foci contained polymorphonuclear leucocytes, degenerated parenchymal cells and nuclear debris. Pathological changes of the spleen were found mostly in the white pulp. In the subchronic experiment of MCPA similar histopathological changes were seen as in the acute experiment. In the liver focal degeneration of parenchymal cells was occasionally observed and the white pulp of the spleen was slightly decreased.

In the present study the histological findings were somewhat different from the MCPA experiments. The changes were most obvious in the kidney and in the intestine whereas with MCPA the

target organ was mainly spleen.

The $LD_{50}$-value for 4-chloro-o-cresol was 1190 mg/kg whereas the corresponding value for MCPA was 500 mg/kg. The tissue concentrations in the subchronic experiment of cresol were remarkably lower than the concentrations of MCPA, probably due to poor absorption from the stomach and small intestine.

On the basis of the present results it can be assumed that 4-chloro-o-cresol is not more toxic than MCPA although more extensive studies are needed to verify this point.

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