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PHENOXY-ACID-INDUCED RENAL CHANGES IN THE CHICKEN

I. ULTRASTRUCTURE

By

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The toxicity of certain chlorinated phenoxyacetic acids (2,4-dichloro- and 2,4,5-trichlorophenoxyacetic acids, in the following abbreviated as 2,4-D and 2,4,5-T, respectively) has been studied previously in laboratory and domestic animals (*Björklund & Erne* 1966). The compounds did not produce unequivocal gross or light-microscopic changes in mammals. In broiler chicks, however, characteristic kidney lesions developed upon prolonged exposure to 2,4-D (given at 1,000 p.p.m. in the drinking water; 2,4,5-T was not studied). The kidneys showed a conspicuous enlargement attributable to hypertrophy of the tubular epithelium. The lesions developed, if very young (3 days old) chicks were exposed to the phenoxy acid; animals of 8 weeks or more did not respond.

The purpose of the present work was primarily to elucidate, by means of electron microscopy, the nature of the renal changes induced in chickens by as well 2,4-D as 2,4,5-T on prolonged exposure. In order to check the significance of the age factor a few days old chicks were compared with 8 weeks old chickens. Furthermore, the reversibility of the changes was studied. Biochemical studies of the changes will be reported separately.

EXPERIMENTAL

Animals and materials

Forty-five day-old broiler chicks (Cornish × W.P.R.) of either sex were purchased from a breeder and reared at the National

Veterinary Institute according to current Swedish broiler breeding practice.

2,4-D. A commercial formulation containing the triethanolamine salt of 2,4-D (without a wetting agent; Regulan DL, Plantex) was diluted with water to contain 1,000 mg of 2,4-D per l.

2,4,5-T. An aqueous solution of 2,4,5-T triethanolamine salt was prepared from technical grade 2,4,5-T and analytical grade triethanolamine to contain 1,000 mg of 2,4,5-T per l.

Experimental design

Forty-five day-old broiler chicks were randomly assigned to 3 groups of 15 animals each: A, receiving from the fifth day of life for up to 7 months 2,4-D (1,000 p.p.m.) in the drinking water, B, receiving 2,4,5-T at the same rate, and C, serving as a control group. (Before the experiment started 2 chicks of group A died.) From 8 weeks of age 2 of the controls were also given 2,4-D (1,000 p.p.m.) in the drinking water for up to 7 months (Group D).

In order to study the reversibility of the phenoxy-acid-induced effects the administration of the acids to 2 chickens each (1 ♂ and 1 ♀) of Groups A and B was interrupted after 20 weeks of exposure and the birds were kept for 8 weeks before sacrifice.

At intervals, animals from each group were sacrificed (Table 1).

Pathological methods

The animals were killed by ether narcosis or by decapitation. The abdominal and thoracic cavities were opened immediately and kidney tissue was secured for electron-microscopic examination. The animals were necropsied and tissue samples taken for light microscopy and, in a few instances, phenoxy acid determination.

Dead animals were necropsied as soon as possible after death. The tissues were examined light-microscopically.

Materials for light microscopy were fixed in 10 % aqueous formaldehyde. Frozen sections were stained with scarlet red for fat. For paraffin-embedded sections Ehrlich's haematoxylin and eosin, van Gieson's picrofuchsin, Alcian blue-PAS and Gomori's silver stain were routinely used.

Determination of phenoxy acids

Selected tissues from a few of the treated animals were analyzed for 2,4-D and 2,4,5-T according to the previously described method (*Erne* 1966 a).

Electron microscopy

Small pieces of kidney cortex, cut out with a razor blade, were fixed either in 2 % osmium tetroxide for 1 hr. (*Millonig* 1961) or in 3 % cacodylate-buffered glutaraldehyde for 4 hrs. followed by osmium tetroxide for 2 hrs. (*Sabatini et al.* 1963). After dehydration the samples were embedded in Epon (*Luft* 1961) and sectioned on an LKB Ultrotome ultramicrotome with glass knives to give large sections of about 1 μm thickness. After staining in buffered toluidine blue (*Björkman* 1962) suitable regions were located in the light microscope, the blocks were re-trimmed and 60—100-nm sections prepared, collected on uncoated copper grids and impregnated with uranyl acetate (*Watson* 1958) or lead citrate (*Reynolds* 1963).

For electron microscopy a Siemens Elmiskop I operated at 60 kv was used. The primary linear magnification was 2,100—11,400.

RESULTS

Clinical signs of toxicity

The general condition of the treated chicks deteriorated during the experiment. The animals became less agile and were often seen sitting with closed eyes. The food and water intakes and the growth rate were lowered compared with the controls (cf. body weight data of Table 1). Seven of the treated animals (3 of Group A and 4 of Group B) died or were killed in a moribund state during the first 4 weeks of the experiment (Table 1), general weakness being the sole clinical sign observed.

Phenoxy acid levels

The tissue levels of 2,4-D and 2,4,5-T observed in some of the treated animals are given in Table 2.

Gross changes

Gross tissue changes were observed mainly in the kidneys of the treated animals. In all animals receiving 2,4-D or 2,4,5-T

Table 1. Experimental data for broiler chickens fed 2,4-D or 2,4,5 T in drinking water (Groups A, B and D) and for controls (Group C).

Animal no.	Sex	Exposure days	Dead (D) or killed (K)	Body weight at death g	Organ weights				Kidney-liver ratio
					kidneys		liver		
					g	per cent[1]	g	per cent[1]	
Group A. 2,4-D (1,000 p.p.m. in drinking water) from 5th day of life									
O 1254/67	♀	14	D	60	2.28	3.8	2.79	4.6	0.82
1257	♂	15	D	65	3.40	5.2	2.63	4.1	1.29
1272	♀	16	K	79	3.15	4.0	3.50	4.4	0.90
1273	♂	16	K	78	2.40	3.1	2.73	3.5	0.88
1297	♂	18	K	67	2.35	3.5	2.42	3.6	0.97
1354	♀	28	K[3]	98	5.79	5.9	4.09	4.2	1.42
2184	♂	104	K	1310	21.9	1.7	21.8	1.7	1.01
2185	♀	104	K	1070	18.7	1.8	20.6	1.9	0.91
2631	♂	141	K	1580	18.1	1.2	25.4	1.6	0.71
2666	♀	143	K	1400	18.7	1.3	24.1	1.7	0.78
2667	♂	143	K	1660	26.6	1.6	29.9	1.8	0.89
179/68	♂	201[2]	K	2370	12.0	0.51	24.2	1.0	0.50
180	♀	201[2]	K	1570	10.0	0.64	39.3	2.5	0.25
Group B. 2,4,5-T (1,000 p.p.m. in drinking water) from 5th day of life									
1255/67	♀	14	K[3]	49	1.60	3.3	2.25	4.6	0.71
1271	♂	16	D	60	4.50	7.5	2.80	4.7	1.60
1274	♂	16	K	63	2.70	4.3	2.80	4.0	0.97
1275	♀	16	K	65	3.50	5.4	3.50	5.4	1.00
1295	♂	18	K[3]	49	2.28	4.7	1.90	3.9	1.20
1296	♂	18	D	59	3.41	5.8	2.40	4.1	1.42
1353	♂	28	K	128	5.70	4.5	4.89	3.8	1.17
2182	♀	104	K	985	24.7	2.5	38.2	3.9	0.65
2183	♀	104	K	945	21.7	2.3	22.7	2.4	0.96
2632	♂	141	K	1690	20.7	1.2	26.7	1.6	0.78
2668	♂	143	K	1430	20.7	1.4	30.0	2.1	0.69
2669	♀	143	K	1150	14.4	1.3	23.2	2.0	0.62
3180	♀	201	K	1370	23.3	1.7	44.1	3.2	0.53
181/68	♂	201[2]	K	2250	10.5	0.45	34.0	1.5	0.31
182	♀	201[2]	K	1680	12.4	0.74	47.6	2.8	0.26

(To be continued)

until death or sacrifice, the kidneys were spectacularly enlarged and had the same pale appearance and firm consistency as in previous experiments with 2,4-D (Björklund & Erne 1966). The kidney weight increase is apparent from Table 1. The collecting tubules and ureters contained ample amounts of whitish excreta. Interestingly, gouty deposits were not noted in any treated animal, neither in the kidneys nor in the serous membranes or the joints.

T a b l e 1 (continued).

Animal no.	Sex	Exposure days	Dead (D) or killed (K)	Body weight at death g	kidneys g	kidneys per cent[1]	liver g	liver per cent[1]	Kidney-liver ratio
Group C. Controls[4])									
O 1256/67	♀	(14)	K	142	1.51	1.1	4.71	3.3	0.32
1276	♀	(16)	K	155	1.70	1.1	4.78	3.1	0.36
1277	♂	(16)	K	152	1.83	1.2	4.40	2.9	0.42
1355	♀	(28)	K	305	3.40	1.1	8.00	2.6	0.43
1356	♂	(28)	K	388	4.40	1.1	11.70	3.0	0.38
2180	♂	(104)	K	1470	11.6	0.79	23.9	1.6	0.49
2181	♂	(104)	K	1748	12.4	0.71	26.3	1.5	0.47
2630	♂	(141)	K	1970	11.5	0.58	33.5	1.7	0.34
2670	♂	(143)	K	1870	9.8	0.52	31.7	1.7	0.31
2671	♀	(143)	K	1690	9.9	0.59	38.4	2.3	0.26
3179	♂	(201)	K	2310	8.6	0.37	28.8	1.2	0.30
177/68	♂	(201)	K	2300	12.0	0.52	24.4	1.1	0.49
178	♀	(201)	K	2220	12.9	0.58	42.3	1.9	0.30
Group D. 2,4-D (1,000 p.p.m. in drinking water) from 53rd day of life									
3177/67	♂	205	K	2050	20.8	1.0	33.9	1.7	0.61
3178	♀	205	K	1550	20.6	1.3	43.6	2.8	0.47

[1]) of body weight.
[2]) killed 59 days after termination of exposure.
[3]) killed in a moribund state.
[4]) for controls third column denotes number of days in experiment.

In the 4 animals of Groups A and B which were sacrificed 8 weeks after termination of exposure the kidneys were normal in size and appearance (Table 1).

T a b l e 2. Phenoxy acid levels in tissues of broiler chickens fed 2,4-D or 2,4,5-T at 1,000 p.p.m. in the drinking water for 104 days.

Chicken no.	Phenoxy acid	Sex	liver	kidney	lung	stomachs[1]	small intestine[1]	egg (yolk)
O 2184/67	2,4-D	♂	4.0	5.0	3.6	20.0	10.0	—
O 2185/67	2,4-D	♀	6.3	10.0	7.2	28.0	15.0	0.4—2.0
O 2182/67	2,4,5-T	♀	3.5	8.5	8.0	85	18.0	0.8—2.1
O 2183/67	2,4,5-T	♀	2.0	3.3	4.1	—	—	0.7—1.6

[1]) contents.

Light-microscopic changes

   The histological examination was confined to the kidneys. As
in previous studies (*Björklund & Erne*) hypertrophy of the renal
proximal convoluted tubules was observed in the treated ani-
mals, the distal tubules and the collecting tubules being only
more or less dilated. The tubular epithelium was usually more
loose and less stainable than in the control animals. No definite
changes were seen in the glomeruli. The renal changes observed
in the treated animals were of the same type, whether newly
hatched or 8 weeks old birds were used and whether 2,4-D or
2,4,5-T was administered. 2,4,5-T, however, generally seemed to
evoke more profound changes than did 2,4-D (Fig. 1 a-c).
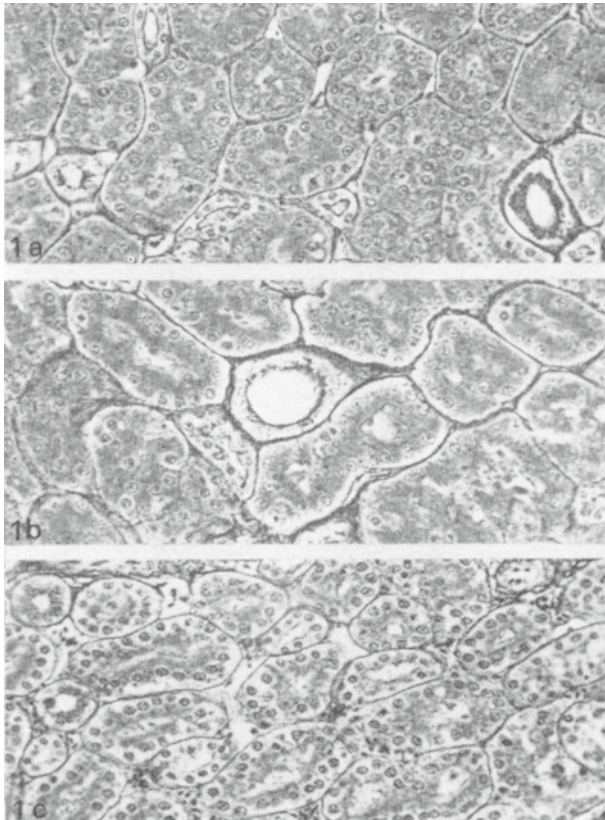
F i g u r e 1 a, b, c. Kidney cortex of broiler chickens fed 2,4-D (a)
or 2,4,5-T (b) for 16 days (from fifth day of life) at 1,000 p.p.m. in
the drinking water. Epithelial hypertrophy of proximal convoluted
tubules. c = control. Gomori's silver stain, × 210.

No light-microscopic abnormalities were observed in the kidneys of the animals sacrificed 8 weeks after termination of exposure.

Electron-microscopic changes

The renal changes seen in all the treated animals were qualitatively similar, although quantitatively differing. Apparently, 2,4,5-T caused more severe changes than did 2,4-D; a strict comparison was difficult, however, because the changes varied in intensity between the animals as well as within the kidneys, in both groups.

Ultrastructural changes were detectable already at the first sampling (after 14 days of exposure), the severity increasing with the exposure period. In the animals sacrificed after an 8-week post-exposure period no significant renal changes were seen.

The glomeruli and the proximal and distal convoluted tubules were subjected to closer examination. The *glomeruli* of the treated animals did not differ appreciably from those of the control animals.

The epithelial cells of the *distal convoluted tubules* were of a more loose appearance than those of the controls, with a dilated and vesicular endoplasmic reticulum.

The most conspicuous changes were seen in the *proximal convoluted tubules* with notable changes in the nuclei and particularly in some of the cytoplasmic organelles.

The size and shape of the *nuclei* and the contents of chromatin, nucleoli and nucleoplasm did not differ between treated and control animals. In the treated chickens nuclear bodies were occasionally seen. Usually there was a central electron-opaque particulate area surrounded by a region of filamentous appearance (Fig. 2, left-hand body), although modifications were frequent (see e.g. Fig. 2, right-hand body, and Fig. 3).

In the cytoplasm changes were apparent in *mitochondria* and *microbodies*. Increased mitochondrial content was more frequent in the treated than in the control animals, as was mitochondrial pleomorphism, with alterations in both size, shape and structure (Figs. 4—7). Elongated mitochondria tended to form circular arrays enclosing various organelles (Fig. 6). After extended exposure periods, mitochondria fused into more or less electron-opaque, structureless bodies appeared (Fig. 7).
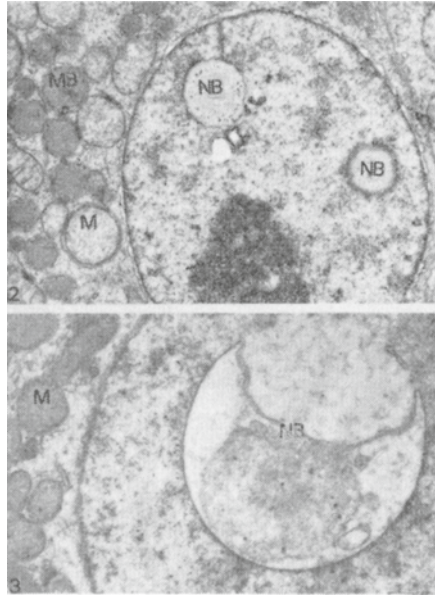
F i g u r e s 2—3. Proximal convoluted tubular cells of broiler chickens
fed 2,4,5-T at 1,000 p.p.m. in the drinking water. NB = nuclear body.
M = mitochondrion. MB = microbody.

Fig. 2. Feeding period 32 days. Two nuclear bodies in nucleus and
several microbodies in cytoplasm. Gluturaldehyde — OsO_4. $\times$ 2,400.

Fig. 3. Feeding period 109 days. Large nuclear body of irregular
structure. Glutaraldehyde — OsO_4. $\times$ 3,450.

The cristae of the inner membrane remained distinctly visible
during the first few weeks of exposure, although irregularities
in orientation appeared early (Fig. 5).

Electron-opaque formations of the microbody type were also
observed more frequently in the treated than in the control ani-
mals (Figs. 2 and 4). The structure was uniform, with a single
membrane enclosing a finely granular matrix which in most
cases contained a crystalloid. In the treated animals the bodies
were of an irregular size and were more often surrounded by a
circular endoplasmic reticulum than in the controls.

As regards *other organelles,* such as lysosomes, endoplasmic
reticulum, Golgi apparatus and ribosomes, and various secretory
products, no significant differences were noted between treated
and control animals.
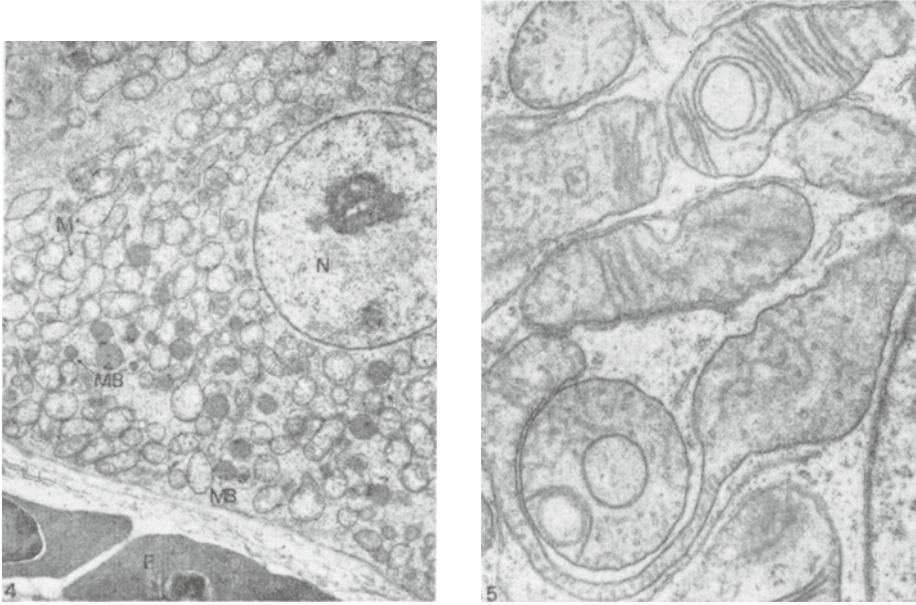
Figures 4—5. Proximal convoluted tubular cells of broiler chickens fed 2,4-D or 2,4,5-T at 1,000 p.p.m. in the drinking water. N = nucleus. M = mitochondrion. MB = microbody. E = erythrocyte.

Fig. 4. Feeding period 32 days, 2,4,5-T. Numerous mitochondria and several microbodies. Glutaraldehyde — OsO_4. $\times$ 1,390.

Fig. 5. Feeding period 16 days, 2,4-D. Enlarged and pleomorphic mitochondria. OsO_4. $\times$ 4,550.

DISCUSSION

The tissue levels of both 2,4-D and 2,4,5-T in the treated animals (Table 2) were largely in accordance with the distribution pattern observed with 2,4-D in previous studies (*Erne* 1966 b). The highest levels of 2,4-D and 2,4,5-T were found in the kidneys, and a distinct excretion of phenoxy acids with the eggs was noticed.

The experimental results further confirm our previous observation that 2,4-D causes kidney enlargement in chickens due to hypertrophy of the tubular epithelium (*Björklund & Erne* 1966). 2,4,5-T was found to evoke similar changes, although often more pronounced. In the previous study the renal lesions developed only when very young chickens were treated, while in the present experiment also 8 weeks old chickens responded. The result might be due to constitutional differences; the animals of the
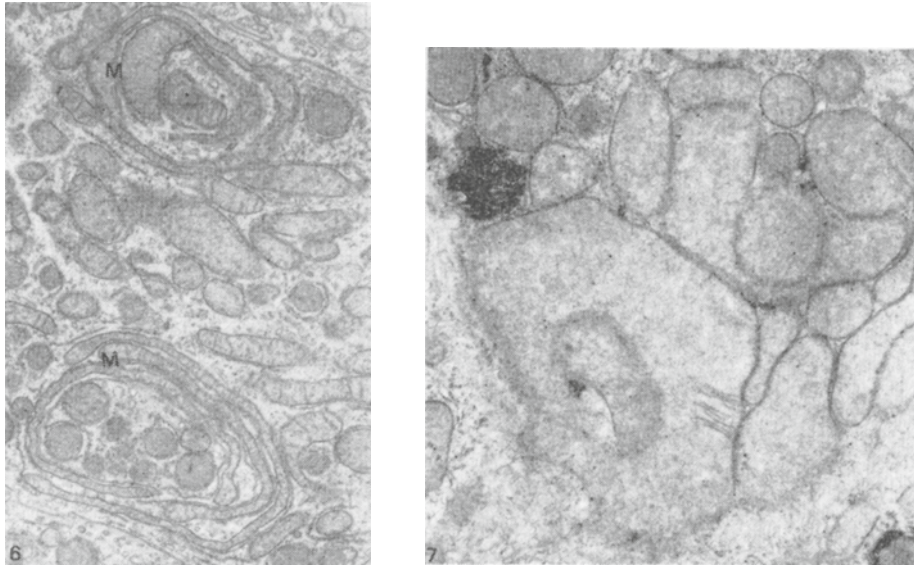
Figures 6—7. Proximal convoluted tubular cells of broiler chickens fed 2,4,5-T at 1,000 p.p.m. in the drinking water. M = mitochondrion.
Fig. 6. Feeding period 14 days. Elongated mitochondria in circular arrays. OsO_4. $\times$ 2,400.
Fig. 7. Feeding period 109 days. Agglomerated, degenerated mitochondria. Glutaraldehyde — OsO_4. $\times$ 4,550.

previous experiments included as well 3-day broiler chickens (Cornish $\times$ W.P.R.), as 8-week White Leghorn chickens and adult New Hampshire hens, whereas the animals of the present work were exclusively broilers. Owing to their higher growth rate the meat-type birds could be expected to be more susceptible to unfavourable environmental factors than the laying-type birds.

On light- as well as electron-microscopic examination, the most spectacular changes were found in the proximal convoluted tubules. This finding is consistent with the result of excretion studies showing phenoxy acids to be tubularly excreted in birds, probably by the proximal convoluted tubules (*Erne & Sperber*). Furthermore, in the excretion studies 2,4,5-T proved to exhibit a markedly higher affinity for the tubular transport system than did 2,4-D and other phenoxy acids examined, an observation which might be related to the (slightly) more pronounced morphological effects of 2,4,5-T, compared with 2,4-D, displayed in the present experiment.

Definite ultrastructural changes were seen in the animals sacrificed after 14 days of exposure. At that time changes were detectable also light-microscopically and grossly. Electron microscopy proved the light-microscopic tubular hypertrophy to be associated with increase in and structural alterations of mitochondria and microbodies. According to *Mölbert* (1968) mitochondrial hypertrophy and hyperplasia usually occur in all instances of enhanced cellular activity, initially also as the first response to a harmful effect. The fine structure of mitochondria has been found to reflect alterations in cellular metabolic activity (*Fawcett* 1966). The phenoxy acid-induced mitochondrial changes observed by us, therefore, might be interpreted as signs of interference with intermediary metabolism (biochemical studies of the changes will be reported separately). For a review of the diversified effects of phenoxy acids on plant metabolism see review by *Audus* (1964). In rat liver mitochondria 2,4-D was shown by *Brody* (1952) to exert a potent uncoupling effect on oxidative phoshorylation. Ultrastructural changes similar to those described by us, including increase and enlargement of mitochondria, were observed in liver cells of rats exposed for extended periods to ethyl α-(p-chlorophenoxy)-isobutyrate (atromide) (*Paget* 1963). (The compound possesses herbicidal properties, but current interest is mainly focused upon its plasma lipid depressing activity). Atromide was also found to induce in rat liver cells the formation of electron-opaque bodies surrounded by a single membrane and without a distinct internal structure. The description is applicable to the microbodies abundantly observed in the kidney cells of our experimental animals. Increased numbers of microbodies have been observed in several pathological conditions, although little is known about their function. They have been regarded variously as precursors to mitochondria and as related to lysosomes (*Fawcett*). A high urate oxidase (uricase) activity has been demonstrated in microbodies which caused *Siebert* (1968) to suggest their participating in urate metabolism. Conceivably, therefore, an increased microbody content of the tubular epithelium would be accompanied by a decreased plasma urate level. The remarkable abscence of any sign of renal gout in the treated animals of the present experiment lends some support to this view. It should be kept in mind, however, that also other factors governing the plasma urate level, such as the tubular transport of urate, might be

affected by phenoxy acids (*Erne & Sperber*). The net result of these effects is difficult to predict.

Nuclear bodies were observed frequently in the treated animals of the present experiment but never in the controls. Such bodies have been found repeatedly in animal cells (*Büttner & Horstmann* 1967, *Büttner* 1968, *Sugimura et al.* 1969). Their function is unknown. According to *Weber et al.* (1964) increased numbers of nuclear bodies of slightly differing shapes have been noticed in a variety of neoplastic cells. These authors consider them to act as "nucleolar organizers" or possibly as "receptor centers for tropic hormons". *Bouteille et al.* (1967) consider nuclear bodies to be related to cellular hyperactivity.

The ultrastructural renal changes induced by 2,4-D and by 2,4,5-T in the chickens of the present experiment were extremely severe in several instances but nevertheless appeared to be of a reversible nature. Irreversible effects induced by phenoxy acids in the animal organism have been described, however, e.g. enzyme inhibition in rat skeletal muscle (*Heene* 1967).

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SUMMARY

Forty-five day-old broiler chicks were equally divided into 3 groups, 2 being fed 2,4-D and 2,4,5-T, respectively, from the fifth day of life for up to 7 months, at 1,000 p.p.m. in the drinking water, and the third serving as a control. From 8 weeks of age 2 of the control animals were fed 2,4-D at the same rate for 7 months.

Seven of the treated animals died, or were killed in a moribund state, during the experiment (Table 1), the survivors showing only slight signs of poisoning such as reduced mobility and food and water intakes.

Animals were sacrificed after varying time intervals (Table 1). The tissue distribution patterns of 2,4-D and 2,4,5-T, as determined in selected tissues of a few of the animals (Table 2), were similar to that observed earlier with 2,4-D.

Both compounds produced qualitatively similar morphological effects, although the action of 2,4,5-T apparently was somewhat more intense.

The predominant necropsy finding was kidney enlargement (Table 1) due to hypertrophy of the proximal convoluted tubular epithelium (Fig. 1).

Electron-microscopically, increased numbers of mitochondria were demonstrated in the tubular cells, with variations in mitochondrial size, shape and structure (Figs. 4—7). The number of microbodies was also definitely increased (Figs. 2 and 4). In the nuclei nuclear bodies were observed (Figs. 2 and 3).

The significance of the findings is discussed.

SAMMANFATTNING

Fenoxiättiksyreframkallade njurförändringar hos kycklingar.

I. Ultrastruktur.

Fyrtiofem daggamla broilerkycklingar fördelades på tre grupper, varav två tillfördes fenoxiättiksyrederivat (2,4-D resp. 2,4,5-T; 1.000 p.p.m. i dricksvattnet) från femte levnadsdagen och under upp till sju månader och den tredje tjänade som kontroll. Från åtta veckors ålder tillfördes även två av kontrolldjuren 2,4-D på samma sätt under sju månader.

Sju av de behandlade djuren dog, eller avlivades i döende tillstånd, under försöket (Tabell 1). De överlevande visade lindriga förgiftningssymtom, såsom nedsatt rörlighet och minskade foder- och vattenintag.

Djuren avlivades efter varierande försökstider (Tabell 1). Vävnadshalterna av 2,4-D och 2,4,5-T, bestämda vid några tillfällen (Tabell 2), antydde distributionsmönster av samma typ som tidigare påvisats för 2,4-D.

Både föreningarna framkallade morfologiska förändringar av likartad typ, men 2,4,5-T syntes vara något mera aktivt.

Obduktionsbilden dominerades av njurförstoring (Tabell 1), betingad av epitelhypertrofi i proximala tubuli contortae (Fig. 1).

Elektronmikroskopiskt påvisades i tubuliepitelet förökning av mitokondrierna med storleks-, form- och strukturförändringar (Fig. 4—7). Dessutom förekom microbodies i starkt förhöjt antal (Fig. 2 och 4). I cellernas kärnor iakttogs nuklearkroppar (Fig. 2 och 3).

Resultaten diskuteras.