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Chief, PB, PMT
Thru: H. J. Richter, Chief, PMT
FROM: Eleanor J. Lloyd, Ph.D. SC-940
Pathology Branch, DPT

January 31, 1969

P-1-69.

SUBJECT: Supplement to Pesticide Petition 9F0743, Daconil (tetrachloroisophthalonitrile) Also called DAC-2787.

Diamond Alkali Company
Painesville, Ohio
(AF 25-202)

Daconil, a fungicide intended primarily for use on potatoes, has been previously reviewed by others. I have been asked to comment on the kidney data from the various animal studies, as results have been conflicting. There have been many toxicity studies on this compound: (1) acute in dogs, rats, and rabbits, (2) a sixteen-week subacute and a 2-year chronic in dogs, ~~xxxxxxxxxxxxxxxx~~ (3) one subacute (3-month) and 3 chronic (2-year, one of these not yet completed) in rats, (4) short ones in mallard ducks, quail, and fish, (5) dermal subacute inunction in rabbits, and (6) 3 reproduction (1 in rabbits and 2 in rats). These have been done by Hazleton Laboratories, Incorporated, in Falls Church, Virginia with the exception of the subacute rat experiment, which was done by International Bio-Research, Inc. of St. Louis, Missouri and Hannover, Germany, and some of the acute experiments, which were done by Hill Top Research Institute, Inc. in Miamiville, Ohio. The material used in testing was in 2 forms: (1) pure DAC-2787, which was used in the rabbit reproduction, rabbit inunction, fish, subacute rat, and most of the acute toxicity studies; (2) a mixture which will henceforth be called the "DAC Mixture" consisting of 35.6% DAC-2787, [redacted] and which was used in the subacute and chronic dog, chronic rat, rat reproduction, and wildfowl studies.

I shall briefly describe each study.

INERT INGREDIENT INFORMATION IS NOT INCLUDED

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I. Acute Toxicity Studies

TABLE I: ACUTE TOXICITY OF DACONIL

Animal	Sex and Number	Laboratory	Compound	Route	Days Observed	LD_{50} in Mg/Kg
Dog	3F	Hazleton	DAC-2787	Oral	7-14	> 5000
Rat	10F	Hazleton	DAC-2787	Stom.Tube	14	>10000
Rat	30M	Hill Top	DAC-2787	Stom.Tube	14	>10000
Rabbit	16	Hill Top	DAC-2787	Epidermal	14	>10000
Rabbit	3	Hill Top	DAC-2787	Eye	7	.3 mg. produced transient conjunctivitis.
Rat	10M	Hazleton	DAC Mixture	Inhalation one hour.	14	> 4.7 mg/liter

II. Dog Feeding Studies - DAC Mixture

Tests in both dog experiments included body weight, food consumption, hematologic studies (hematocrit, hemoglobin, erythrocyte count, and total and differential leukocyte count), urinalysis, and blood chemistry (SGPT, BUN, BSP, and coagulation time). Additional tests performed in the sixteen-week study were blood glucose, protein-bound iodine (PBI), CO_2, SGOT, and serum bilirubin, protein, albumin, Na, K, Ca, and Cl. The only other test performed in the 2-year experiment was the sedimentation rate. In both studies each animal started on the experiment was grossly and microscopically examined.

A. Sixteen-Week Subacute. Beagles, 4 males and 4 females per group, received either 0, 250, 500, or 750 ppm. The only effect noted was a dose-related increase in the PBI range from 5 micrograms % in the controls up to 16.1 at 750 ppm; no changes were seen, however, in the thyroid. In regard to the kidneys, focal tubular epithelial hyperplasia, vacuolization of the proximal tubule, and focal leukocytic infiltration were seen in dogs from all 4 groups, but as these changes were as common in control as treated animals they could not be ascribed to Daconil.

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B. Two-Year Chronic. Beagles, 4 males and 4 females per group, received either 0, 0.15, or 3% in the diet. Effects occurred in (1) liver, (2) kidneys, (3) thyroid, and (4) body weight. A no-effect level was not demonstrated as there were changes at all 3 dosage levels in the liver and kidneys, though a relation to dose was evident, with pathology varying from

mild at 0.15% to moderately severe at 7%. (1) Hepatic abnormalities consisted of portal f
brosis, bile duct proliferation, hepatocytic i regularity, and increased pigment (whether
bile, hemosiderin , or some other was not stated) in hepatocytes and macrophages; severe p
tal cirrhosis was also reported in 1/8 dogs at each of the 2 upper doses. (2) Kidney lesi
were glomerulosclerosis (a change which is either serious or potentially serious, dependin
upon the degree of involvement) and pigment deposition (which is less serious); tubular di
latation and slight tubular epithelial vacuolation were also observed at all 3 doses and a
peared to bear some relation to dose, but as the former was also reported in rats but was
observed in slides from these rats when studied by several FDA pathologists (Drs. H.L.Rich
ardson, R.T.Habermann, F.A.Gross, and myself) and the latter is normal in the pars recta o
the proximal tubule of the dog, it is questionable whether these changes should be regarde
as valid. (3) The thyroid was the site of pigmentation at 3% and 1.5%; the PBI was not det
mined in this study. (4) There was slight loss of weight (an average of 0.7 kg) in 5/8 do
on 3%, contrasting with average gains ranging from 2.4 kg at 1.5% to 10.7 kg at 0% at the
other 3 levels.

III. Subacute and Chronic Rat Feeding Studies

A. Subacute Study, International Bio-Research, Inc. (DAC-2787)

1. Methods. Rats, 10 of each sex per group, received either 0, 0.5, 1.0, 2, 4, or 8 gm
per kilogram of DAC-2787 (97% pure) by stomach tube 5 days a week for 6 or 13 weeks. After 6 weeks,
remaining ones on 8 g/kg were returned to control rations. Tests included body weight, f
consumption, hemograms (hemoglobin, hematocrit, erythrocyte count, and total and differen
leukocyte count), and gross and microscopic examination of rats from each group after 3 w
of treatment, and from the 0, 2, 4, and 8 g/kg groups after 13 weeks.

2. Effects. (1) Loss of weight at 2 g/kg, with depression of growth (not statistically
nificant) at 4. (2) Poor clinical condition and decreased resistance to infection, manif
ted chiefly by increase in incidence and severity of suppurative pneumonitis at 4 and 8 g
effects at the high dose were reversible. (3) 4 deaths at 8 g/kg and 2 at 4.

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3. First Two-Year Experiment Project 200-148

1. Methods. Weanling rats, 35 of each sex per test group and 70 of each sex in the contr group, received either 0, 0.15, 1.5, or 3% of DAC Mixture for periods ranging from 3 to 24 months (in those at 3% the dose was reduced to 0 the 5th day, gradually increased to 3% again by 3 months, at which time half were killed and the remaining half had their dose re duced again to 0 and were killed at 47 weeks). Experimental procedures periodically deter mined were body weight, food consumption, hemograms, coagulation time, PBI, and urinalysis Animals from the 0, 0.15, and 1.5% groups were killed and autopsied after 3, 12, and 24 months, and those from the 3% group after 16 and 47 weeks. A few from each dosage group were examined microscopically at each of these intervals, the total number so examined bei 50/140 on 0%, 30/70 on 0.15%, 14/35 males and 21/35 females on 1.5% and 30/70 on 3%. The slides made from the kidneys of the 3-month rats on 0, 0.15, and 1.5% were studied by the FDA pathologists referred to above.

2. Non-renal Effects. (a) Slight but statistically non-significant decrease in 2-year sur vival in the males at ~~each~~ both dosage levels (20%) in comparison with the controls (38%). (b Dose-related decrease in the PBI in comparison with the controls at all levels of treatme at 12 months, although there was no real difference between treated and control groups at 24 months and the thyroids in all groups at all time intervals appeared to be histologica within normal limits. (c) Growth depression at 3 months at 3 and 1.5%, and at 12 and 24 months at 1.5%. (d) Acanthosis of the forestomach at 3 and 1.5% at 3 months and at 1.5% after 1 and 2 years, but not in the recovery group originally on 3% after 47 weeks. (e) F to moderate liver changes, including 1 case of cirrhosis, at 1.5% after 2 years.

3. Renal Effects.

a. Gross. (1) One striking gross change was a statistically significant absolute and relative (per cent body weight) increase in weight in the treated rats in comparison with the controls. There were absolute increases after 2 years in the 1.5% males (average weight of both kidneys 7.2 grams versus 5.2 grams for the controls). There were also re tive increases in both sexes at all 3 treatment levels after 3 months, at both doses

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the greenish-brown discoloration

(1.5 and 0.15%) in the males and at 1.5% in the females after 12 months, and in both sexes at 1.5% after 2 years. (2) Another important change at 1.5% was roughening of the surfaces in all 4 2-year survivors and in most of the males that died between 1 and 2 years. (3) A third alteration was noted in a majority of the kidneys at all time intervals in the treated groups at levels above 0.15%.

b. Microscopic. (1) Tubular dilatation was reported at all dosage levels (control as well as treatment) and time intervals but appeared more common after 12 months at 3 and 1.5% than at 0.15% and 0%; however, at 24 months it was limited to females in both treated and control groups. When our FDA pathologists (previously cited) examined the slides from the 3-month animals at 0, 0.15, and 1.5% (all doses except 3%) we were unable to detect any true dilatation. (2) We were also unable to find the tubular epithelial degeneration Hazleton stated was present in all 6 3-month females at 1.5% (though we did find slight tubular epithelial vacuolation in 2/6 female controls). Hazleton scientists also reported such degeneration in all 10 3-month rats on 3% and 9/10 on 0.15% and 6/10 on 1.5% at 12 months. In the 2-year rats they also found (3) glomerulosclerosis in a few rats at all 3 levels (including the controls) (4) tubular hyperplasia in 9/14 on 1.5%, 4/5 (all female) on 0.15%, but only 2/20 on 0% (this would thus seem to be an effect of treatment at 1.5% but its predominance in females at the low dose confuses the issue), and (5) more tubular hypertrophy at 1.5% and 0.15% than in the controls. (6) Chronic nephritis was said to be present in nearly all the 2-year animals and was apparently as common in controls as treated groups. The criteria for diagnosis, however, were not given, which is another source of confusion, as some of the other changes listed above (tubular dilatation and hyperplasia and glomerular sclerosis) are generally considered parts of this lesion. (7) Tubular pigmentation was noted in 2-year rats from all groups (including the controls), though gross discoloration was seen only in treated groups.

c. Interpretation. From my own observations, I do not believe there are any significant alterations in the kidneys of the rats treated 3 months below the 3% level; though the relative weights of these organs were increased this is not necessarily of significance in a young and rapidly growing animal. It would seem, however, that there probably were toxic effects at 3%. Because of the relatively small number of kidneys studied microscopically

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(only 4[illegible] from the 2-year rats of the original 3[illegible]0 were thus examined), the fact that those which were examined were from survivors rather than rats which had died (and were thus presumably deleteriously affected by something, especially since most of the 1.5% male kidneys from non-survivors were grossly abnormal), and the failure of the changes found to bear any consistent relation to dose, it is difficult to be sure of the levels at which Daconil produced toxicity. On the other hand, it is clear that the compound did result in chronic renal pathology, particularly after being fed for 2 years, because of the striking absolute and relative increases in kidney weight at 1.5%, the gross roughening of the capsular surfaces in the 2-year 1.5% males (most commonly indicative of chronic nephritis), the greenish discoloration in the test animals, and the higher incidence of tubular hyperplasia and hypertrophy (features also present in chronic nephritis) in the treated group than in the control. If more kidneys had been studied microscopically, it is possible that tubular dilatation and glomerulosclerosis, other characteristic changes in chronic nephritis, might have shown a more consistent relation to dose. However, the evidence presented shows that Daconil induced renal pathology, probably an increase in chronic nephritis (a common spontaneous lesion in aging rats that can always be found in a certain number of untreated controls) possibly a somewhat different disease related to the pigmentation (as Hazleton suggests), after being fed for 1 to 2 years at 1.5% and possibly also at 0.15%.

C. Second Two-Year Experiment, Project 200-154

1. Methods. Weanling rats divided into 2 groups, each composed of 35 of each sex, received either 0 or 0.5% of DAC Mixture in the diet for periods up to 2 years. Tests were similar to those in the first 2-year experiment, except that the only organs studied microscopically were kidney, thyroid, forestomach, and lung, and that there is no record of microscopic examination of any animal treated longer than 1 year. A total of 20/70 controls and 20/70 test rats were thus examined, 10 from each group after 3 months and 10 after 12 months.

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2. Non-renal Effect. Decreased rate of growth.

3. Renal Effects of Treatment.

a. Gross. Increased relative weight at 3, 12, and 24 months, significantly increas absolute weight in the males at 12 months, roughening of the surfaces in half the males and several females after 1-2 years, and, at all time intervals, the greenish discoloration previously noted (the last chiefly in the males).

b. Microscopic. Increased chronic nephritis in the test rats after both 3 and 12 months, and tubular epithelial vacuolation after 1 year in the test but not in the control group.

c. Interpretation. As renal pathology was greater at 0.5% than at 0%, the effect must be attributable to Daconil.

Experimental procedures which are being periodically determined during life are body weights, food consumption, hemograms, urinalysis, and blood chemistry (sugar, and SGOT).

D. Third Two-Year Experiment, Project 200-205

TABLE II

VACUOLATION OF PROXIMAL TUBULE OF RATS TREATED THREE MONTHS

PPM Diet	Micro. Sec'd. M	Micro. Sec'd. F	MALES Hazleton No.	MALES Hazleton Grade	MALES Long No.	MALES Long Grade	FEMALES Hazleton No.	FEMALES Hazleton Grade	FEMALES Long No.	FEMALES Long Grade
0	7	7	0	0	0	0	0	0	0	0
4	7	7	0	0	0	0	3	V.Sl.	3	Slight
10	7	7	2	Min.	0	0	6	V.Sl.	5	Mod.
20	7	7	2	Min.	1	Min.	7	V.Sl.	5	Slight
30	7	7	3	Min.	2	Min.	6	Slight	4	Slight
40	7	7	6	Slight	1	Min.	6	Slight	6	Sl.-Mod.
60	7	7	6	Slight	1	Min.	7	Mod.	7	Sl.-Mod.

Abbreviations
Min. - minimal
V. Sl. - very slight
Mod. - moderate
Sl. - slight

1. Methods.

This study is at present in progress. Groups composed of 50 male and 50 female are being fed either 0, 4, 10, 20, 30, 40, or 60 ppm of DAC Mixture. After 3 mont 15 of each sex per group were killed and autopsied, and 7 of each 15 sectioned and died microscopically.

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2. Effects at Three Months.

The only changes noted thus far have been in the kidneys. The slides made f the kidneys of these 3-month animals have been studied by the group of FDA patho

to whom reference has already been made. We agree with Hazleton that the distal segment (pars recta) of the proximal tubule of manganeated animals but no controls showed marked degrees of degeneration ranging from swelling and vacuolation of the cytoplasm of the lining epithelial cells to luminal deposition of protein and (in the most severe cases) epithelial cell necrosis. We disagree with Hazleton, however, in the distribution and severity of this lesion among the rats in the various groups. Hazleton reported an effect related to dose but not to sex (though its data show that more females than males were affected at the lower levels) present at all levels of treatment, with 13/14 rats at 60 ppm but only 3/14 at 4 ppm revealing the lesion. After looking at the slides, we on the other hand found it to be practically limited to females and to bear a slight relation to dose (7/7 females with an average grade of slight to moderate at 60 ppm but only 3/7 with an average grade of slight at 4 ppm).

It is interesting that Hazleton commented that the tubular dilatation noted in the preceding rat experiments was not evident here. I agree that this is absent here. This is not surprising, as these sections have the typical features of tissue fixed in Zenker's solution, which characteristically shows the convoluted tubule to have a small lumen and tall pyramidal lining cells in contradistinction to the more generally used formalin fixation (used for the kidney sections I looked at from Project 200-148) in which the tubules may appear dilated because of a larger lumen and flatter lining epithelial cells.

In summary, it seems, unfortunately, that Daconil caused mild changes in the female kidneys at a dose as low as 4 ppm and in the male at one as low as 20 ppm when fed in the diet for 3 months. What the effect will be after longer periods remains to be seen.

IV. Short Toxicity Studies in Other Animals

A. Rabbit, Skin.

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TABLE III

DAC-2787 in Mg/Kg	Number of Rabbits → Intact Skin Tot.	Intact Skin Micro.	Abraded Skin Tot.	Abraded Skin Micro.	Number of Inunctions	Deaths	Effect
0	5	5	5	5	15(1 daily except between days 5&6 and 10 & 11)	0	None
500	10	5	10	5		0	Skin
1000	10	5	10	5		1	Skin

1. Methods.

DAC-2787 was applied by inunction to the intact or abraded skin of male and female albino rabbits according to the schedule above. At intervals the animals were weighed, and hemograms and urinalyses ~~xxxxxxxx~~ obtained. At the termination of the study the rabbits were killed and autopsied. Microscopic examination in the 0 and 1000 mg/kg groups limited to skin, liver, and kidney, and the 500 mg/kg group to skin only.

2. Effects of Treatment.

(a) Death after 8 inunctions of one high-dose animal. (b) Skin changes of great severity on abraded areas but present at both levels of treatment; they were characterized grossly by desquamation and thickening, and microscopically by moderate acanthosis and hyperkeratosis, slight to moderate leukocytic infiltration, and occasional focal parakeratosis. (c) There was some spontaneous nephritis at all 3 levels. As the incidence and severity were no greater in treated than in control groups, however, the lesion could not be attributed to Daconil.

B. Fish, Aqueous Toxicity

Fish were tested for 96 hours. The results in Table IV below show that DAC-2787 is many times less toxic than DDT.

TABLE IV: TOXICITY OF DAC-2787 IN FISH

Fish	LC50 in Parts per Billion p,p'-DDT	DAC-2787
Rainbow Trout (at 55°F)	0.48	250
Bluegill Sunfish (at 75°F)	1.30	286
Channel Catfish (at 75°F)	18.00	430

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C. Birds. The LC_{50} of DAC-Mixture administered in the diet for 5 days proved to be over 21,500 ppm for mallard ducks, and over 1780 ppm (in another study it was 1020 ppm) for quail.

V. Reproduction Studies

A. Rabbits

TABLE V: REPRODUCTION STUDY IN RABBITS

Group	Does	DAC-2787 in Mg/Kg by Capsule Days 8,9	Days 10-16	Maternal Deaths	Fetal Skeletons Cleared
1	8	0	0	0	29
2	8	180(0.5%)	62.5(0.5%)	2	19
3	8	375(1.0%)	31.25(0.25%)	3	12

Each doe was mated with a buck and dosed on the days of gestation shown above. Necropsies were performed on the fetuses delivered by Caesarean section on the 22nd and 23rd days and theirs mothers. Effects of treatment were: (1) decreased food consumption, (2) weakness, (3) 5 maternal deaths in the treated groups, and (4) increased fetal deaths in the 2 treated groups. Factors 2,3, and 4 can reasonably be attributed to the poor food consumption. No anatomical abnormalities were found in the fetuses.

B. First Rat Study, Project 200-150

TABLE VI: FIRST RAT REPRODUCTION STUDY OF DAC-MIXTURE

Group	% Fed	No. Parents M	F	Matings	Autopsies Gross Only	Microscopic	Effects Parents	Offspring
1	0	10	20	2	Excess F_1, All F_3	20 F_3	No	No
2	0.15	10	20	2	All F_3	20 F_3	Yes	Yes
3	1.50	10	20	2	All F_3	20 F_3	Yes	Yes
4	3-2	10	20	1	All F_1, 10 F_1	0	Yes	Yes

1. Methods;

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[redacted] A 3-generation oral rat reproductive study using DAC Mixture was carried out according to the schedule in Table VI. Feeding of the test compound was to both parents.

in all 3 generations and was begun several weeks before the first parental(P_1) generation was mated. The dose in Groups 3 and 4 had to be reduced to 0 after the first 3 days, after which time it was raised in Group 3 to reach the original level of 1.5% by 8 weeks, and in Group 4 to reach 2% by 10 weeks. Before the second mating of the P_1 generation, Group 4 was discontinued.

2. Effects of Daconil.

a. Parents. (1) Decreased growth at all levels of treatment. (2) Inflammation of eyelids and roughening of fur at 2 and 1.5%. (3) Enlargement with roughening and pitting of surfaces of P_3 1.5% kidneys, with green or brown gross renal discoloration in 2% P_1 kidneys and at both doses (1.5 and 0.15%) in P_3 kidneys. (4) Distension of cecum with softened feces in 2% P_1's and at both doses for P_3's. (5) Gross thickening of gastric wall in P_1's at 2%.

b. Offspring. (1) Depression of growth at all dosage levels. (2) No increase in malformations. (3) Focal tubular epithelial vacuolation in a few F_3 kidneys at both 1.5 and 0.15%. (4) Gastric and esophageal acanthosis and hyperkeratosis at 1.5 and 0.15%. (5) Hunching and inflammation of eyelids at all levels of treatment but with a positive relation to dose.

C. Second Rat Study, Project 200-155

1. Methods. This experiment was performed in a manner similar to the first, except that there were only 2 groups (0% and 0.5%) instead of 4. Autopsies were on all P_3 rats and on representative animals from the P_2 group and the F_{1b} and the F_{3b} weanlings.

2. Effects of Treatment.

a. Parents. (1) Growth depression. (2) Changes which were apparently limited to the P_2 males were enlargement and yellow-green discoloration of the kidneys. (3) Changes which were found predominantly in the P_3 group included hunching, yellow ears, inflamed eyelids, rough and stained fur, and soft and mucoid feces.

b. Offspring. Depression of growth.

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DISCUSSION AND SUMMARY

It is clear from the above data that Daconil has been extensively studied in dogs, rats, rabbits, and even fish and birds. The acute toxicity is low. The oral LD_{50} for both dogs and rats is greater than 200,000 ppm (over 5000 and 10000 mg/kg, respectively), and 3 species of fish exposed for 96 hours were able to tolerate 23 to 521 times the amount of DDT. Chronically the compound also seems to be well tolerated as far as survival is concerned, as dogs have ingested amounts as high as 3% of the diet (30,000 ppm, a fantastic dose for this animal) with no fatalities, and rats amounts as high as 1.5% (15,000 ppm) without a statistically significant increase in mortality in comparison with untreated controls (though there was an increase at both 1.5 and 0.15%) for 2 years; a similar good tolerance was evident in the two rat reproduction studies. However, although survival seems to have been little affected, pathological changes have been demonstrated not only at these high levels but at lower ones as well. Changes in the lungs, liver, thyroid, stomach, and esophagus have presented no real problems. The only experiment in which the lungs were affected was the 3-month oral toxicity (stomach tube) rat ~~xxxxxx~~ study done by International Bio-Research, Inc. In this there was an increase in the incidence and severity of suppurative pneumonia at the very high levels of 40000 and 20,000 ppm, but no such effect was seen at 20,000. As for the thyroid, it was apparently affected only in the dog and in this animal only after 2 years and at 3 and 1.5%, at which levels it was found to contain a greenish-brown pigment. It is interesting that in the 3-month dog study the PBI (which was not tested in the chronic experiment) showed a dose-related increase; this may not have been related to the thyroid pigmentation, however, as this test showed erratic changes when performed at several intervals in the first chronic rat study (Project 200-148). The liver in the rat revealed only mild to moderate alterations (except for 1 case cirrhosis) and these were limited to the high dose (1.5%) group and seen only after 2 years. In the dog, on the other hand, after 2 years on Daconil dose-related alterations were found at all levels of dosage, ranging from mild at 0.15% to moderately severe (with severe portal cirrhosis in 2, at 3%. Acanthosis of the forestomach was not reported in the dog but was prominent

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in the rat in both the chronic toxicity and reproductive experiments. In the former, it was found at levels above 0.15% as early as 3 months, as well as at 1 and 2 years; in the latter it was seen in the third generation offspring (in which the esophagus was similarly involved) at 0.15% also. Though the parents in the reproductive studies were not examined microscopically, it was probably present in them, likewise, at 3%, as the walls of their stomachs were thickened. The absence of the lesion in the 3% rats which were taken off the compound and allowed to recuperate for several weeks shows that it is reversible.

In contrast to the organs discussed in the preceding paragraph, changes in the kidneys, which have been found in both dogs and rats (but not rabbits) in almost every experiment, do present certain problems. There is little doubt that Daconil induced renal pathology in the dog at all levels, as changes consisting of glomerular sclerosis, deposition of pigment within the tubules, and possibly other tubular changes varied directly in degree and incidence with the dose, ranging from mild at 0.15% to moderately severe at 3%, and were virtually absent at 0%. It is also clear that the kidneys of the rats were deleteriously affected by this fungicide: what is not clear are the levels involved and the exact nature of the lesions. There are actually 2 problems, a subacute and a chronic. In the subacute, the problem lies in the wide discrepancy in the levels at which effects were seen among the three 3-month experiments. In the one done by International Bio-Research, Inc., no lesions were produced at levels as high as 8 grams/kg (8%), while degeneration, with or without vacuolation, of the tubular epithelium was seen in Hazleton Project 200-148 at 3% (50,000 ppm) but not below), but in Hazleton Project 200-205 at doses as low as 4 ppm in females and 20 ppm in males. In the females there is no doubt that this vacuolation degeneration was an abnormal change induced by Daconil, as we at FDA found upon examining the slides histologically that there was a definite increase with increasing dose. As for the males, as we found it to be much less extensive and severe than did the Hazleton pathologists in the slides we had the opportunity to examine, it is only natural for me to doubt it was as extensive as they stated in the animals we did not study microscopically (all ten 3-month rats of both sexes at 3% in Project 200-148, most of the

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yearlings at 1.5% and 0.15% in Project 200-148, all 5 male and 1 female yearlings at 0.5% in Project 200-154, and some of the offspring at both 1.5 and 0.15% in the first reproductive experiment). However, since we know that the lesion was present in at least one group of treated animals which we examined, it is reasonable to assume that it was also present in some (if not all) of the others in which it was reported. Therefore, if it was this widespread in the treated animals (even though bearing no clear relation to dose) and only rare in controls, we may assume that it was probably induced by the treatment in the males as well as the females. However, if the severity of the lesion was no greater in the animals I did not examine than in those I did, I would not consider it serious. If this was produced by Daconil it is curious that it was not seen in the study done by International Bio-Research. The explanation may lie in the fact that 97% pure DAC-2787 was used, whereas in the Hazleton studies DAC Mixture (94% DAC-2787) was used. This, of course, would mean that the tubular degeneration was not due to DAC-2787 but to one of the other compounds in the mixture. While this does not explain the dosage discrepancy between Projects 200-148 and 200-205 (both of which used DAC Mixture), it does suggest another possibility, namely, that the composition of DAC Mixture may not have been uniform at all times.

The mild tubular degenerative changes found in the subacute rat study, while important, are not as serious as the changes which were found in the kidneys of the rats treated for 2 years, though the two may have been related. The chronic lesion was characterized (1) grossly by enlargement, greenish-brown discoloration, and pitting and roughening (granularity) of the surface, and (2) microscopically by deposition of pigment (called lipofuscin by Hazleton though whether the special tests necessary for identification were done was not stated) within the epithelium of hyperplastic, hypertrophied, and dilated tubules. Hazleton reported that it was present at the high dose (1.5%) in the 2-year rats in Project 200-148. The problem lies in differentiating this lesion from chronic nephritis, a common spontaneous lesion in old rats characterized by all the features seen in this entity (with the exception of the pigment) plus glomerulosclerosis. Though Hazleton stated that chronic nephritis was as severe in the controls as in the treated groups,

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the gross data do not support this, as, while there was no mention of the controls, the kidneys in the majority of animals (especially the males) in all treatment groups over 0.15% showed discoloration, relative and/or absolute enlargement, and a rough surface. Furthermore, similar gross changes were also noted in the parents (of both sexes, but predominantly males, as in the 2 chronic toxicity experiments) at 1.5% (with discoloration only seen at 0.5%) in in the rat reproduction studies: as these animals were probably not over 7 or 8 months of age when killed, finding these changes in them is even more serious than finding them in the older ones, as it indicates that breeding accelerated their development. Although the gross data show that the 1.5 and 0.5% rats had abnormal kidneys, detailed microscopic study of all groups is necessary to elucidate the exact nature and extent of the lesions, and determine whether the 0.15% level was also affected, and whether the changes at all treatment levels varied significantly from those in the control group to be considered effects of the compound administered. For this, it is necessary to examine a large number of kidneys from all groups, as small but important microscopic changes may not be apparent grossly, and when the gross findings indicate that a common spontaneous disease such as chronic nephritis is clearly present, it is only by such detailed microscopic study of many kidneys that it is possible to determine whether there is a significant difference in incidence and severity among the various dosage levels. (In this case, the chemical nature and pathogenesis of the pigmentation and its relation to the other renal pathology should also be explored, as abnormal pigmentation is an uncommon finding. Its presence in the liver, kidneys, and thyroids of the dogs treated for 2 years makes it even more significant, even leading to the suspicion that it might have been stored Daconil.) Unfortunately, as Table VII demonstrates, enough kidneys were not sectioned and studied microscopically here to answer these questions. Ten per group may be adequate for a 3-month segment of a longer study if the animals are clinically and grossly normal, but 20/140 or 10/70 from the 2-year group in which spontaneous disease can be expected are totally inadequate (and the few kidneys that were so examined were shown that the lesions mentioned earlier were scattered through both treated and control groups), not to mention the fact that none were examined microscopically after 2 years in Project 200-154!

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Moreover, the report reveals that the only 2-year kidneys thus examined were from the few survivors, whereas the gross lesions noted were just as common in the more numerous non-survivors, (those dying between 1 and 2 years). These non-survivors should have also been studied. Organs from dead animals are often discarded because of autolysis but this is a mistake when tumors or chronic lesions are present, as both generally contain so much fibrous tissue (which autolyzes more slowly than epithelium) that enough change can usually be detected to make a diagnosis which would be missed by gross inspection alone. Moreover, when there is only slight autolysis, almost any pathologic change can be read. To summarize, when gross lesions are present in rodents, organs from a majority of the animals or all the remaining animals in a 2-year study should be studied microscopically. If a choice must be made between using one or two-year clinically and grossly normal animals, the yearlings can be eliminated, as lesions are less apt to be found at this period than earlier or later. Of course, at all periods, unless there is advanced autolysis, all gross lesions require microscopic examination.

TABLE VII: NUMBER OF RATS EXAMINED MICROSCOPICALLY IN CHRONIC TOXICITY STUDIES

Project	Number Started	Examined Microscopically 3 Months	12 Mos.	24 Mos.	Totals Examin.	Total Not Examined
200-148						
0.0%	140	10	20	20	50	90
0.15%	70	10	10	10	30	40
1.5%	70	11	10	14	35	35
3.0%	70	10	20	Discontinued	30	40
Totals	350	41	60	44	145	205
200-154						
0.0%	70	10	10	0	20	50
0.5%	70	10	10	0	20	50
Totals	140	20	20	0	40	100

In summary, while the gross and microscopic changes found show that drug-related pathology developed at levels of 1.5% and 0.5% in the kidneys of virgin rats (predominantly male) fed Daconil for 2 years in chronic toxicity studies 200-148 and 200-154 and in those of male and female parents treated similarly for much shorter periods in 2 rat reproduction studies, an insufficient number of kidneys were studied microscopically to determine the exact nature of

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001109

the lesion and whether it was also present at the lowest dose, 0.15%. In a third chronic toxicity feeding study in rats which is now in progress (Project 200-205), dose-related vacuolation degeneration of the pars recta of the proximal renal tubule was found after 3 months in females at much lower doses ranging from 4 to 60 ppm, with a few males from 20 to 60 ppm also affected. In view of these changes and the prominent renal pathology also demonstrated in the dog, a thorough microscopic investigation will be necessary to establish a minimum effect level, if there is one, for the chronically treated rats. A similar adequate investigation of the other organs to rule out any other adverse affect is also desirable; because of alterations found in the stomach and esophagus at higher levels in the rat, and in the dog thyroid (with associated changes in the PBI), these organs should also be studied extensively microscopically. Much time has been wasted in the investigation of Daconil simply because of the failure to examine enough animals microscopically; a thorough job is necessary this time. I therefore recommend that the following be done on Project 200-205. (1) The kidneys, esophagi, stomachs, thyroids, livers, and all other grossly abnormal organs of all rats surviving the 2-year experimental period and of all those dying (except the ones markedly autolysed) at any period should be examined microscopically. (2) All other organs from at least 10 rats of each sex per dosage level should be similarly sectioned and studied microscopically. (3) The chemical nature of the pigment prominent in both dogs and rats should be investigated. (4) In case Daconil should prove to have no chronic effects in rats at these low levels, a similar microscopic investigation of the first chronic rat feeding study (Project 200-143) and possibly the second one (Project 200-151) also will be necessary to determine whether the compound induced organic effects at 0.15% (1500 ppm). (5) The present format for reporting is good, except that we would like listing and grading of individual lesions in individual animals in addition to the group evaluations now being submitted. (6) The criteria for diagnosing chronic nephritis should be stated, as these can differ somewhat from one pathologist to another.

cc: ① Dr Johnson
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Dr Blumenthal to State who he wants copies for.

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