

SUBJECT: DACONIL (Chlorothal: 2,4,5,6-Trichloroisophthalonitrile)
and 4-Hydroxy Metabolite (4-Hydroxy-2,5,6-Trichloroisophthalonitrile)

Dec. 6, 1974

TO: Dr. Clara Williams
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Registration Division

Pesticide Petition 2F1230

Diamond Shamrock Chemical Company
300 Union Commerce Building
Cleveland, Ohio 44115

Related Petitions: 7G0516, 7F0539, 3F0734, 1F1024

This is my 8th review of Daconil, the previous ones being dated as follows: (1) January 31, 1969, (2) May 16, 1961, (3) August 8, 1963, (4) September 16, 1967, (5) February 15, 1973, (6) February 28, 1974, and (7) July 3, 1974. The present review is concerned with (a) new studies we requested on the 4-hydroxy metabolite: (1) 90-day dog feeding, (2) 120-day rat feeding, and (3) 3-generation rat reproduction, and (b) further consideration of 2-year rat feeding study 200-205 on the parent compound, which has been previously reviewed and is the subject of considerable controversy. The studies on the metabolite were done by Bio/Tox Research Laboratories, Inc., 553 North Broadway, Spencerville, Ohio 45887. Rat study 200-205 on Daconil was done by Hazleton Laboratories, Incorporated, Falls Church, Virginia.

I. Studies on 4-Hydroxy Metabolite (DAC-3701)

A. Thirteen-Week (Ninety-Day) Subacute Dog Feeding Study.

PPM Fed	Dogs Started	Weight Loss	Deaths	Liver Damage		Renal Tubular Damage			Autolysis	
				Bile Stasis	Necrosis	Degen.	Vacuol.		Liver	Kidney
						M	F			
0	8	No	0	0	0	0	0	1	0	0
50	8	No	0	0	0	0	1	1	0	0
100	8	No	0	0	2(slight)	0	1	2	0	0
200	8	Yes	8	7	4(1 focal)	4	?	?	4	4

Methods.

Beagles 4.5 to 5.5 months of age, 4 of each sex per group, were fed DAC-3701 at levels of 0, 50, 100, or 200 ppm in the diet, which consisted of Wayne Dog Food, for 13 weeks. Testing procedures during life consisted of weighing, physical examination, food consumption, daily observation, hematology (erythrocyte count, total and differential leukocyte count, hematocrit, and hemoglobin), biochemistry (blood glucose and urea nitrogen, SGOT, SGPT, and serum bilirubin, calcium, sodium, potassium, chloride, and alkaline phosphatase), and urinalysis. Laboratory studies were done initially, and after 4 and 13 weeks. Autopsies were performed on all dogs, and organ weights were obtained on survivors. Microscopic examination was done on (1) thyroid, heart, liver, gallbladder, spleen, kidneys, adrenals, pancreas, stomach, small intestine, colon, mesenteric lymph node, urinary bladder, testes, ovaries, and

bone marrow from 0 and 200 ppm dogs, and (2) liver, kidneys, and unusual gross lesions from those on 100 and 50 ppm.

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Effects.

1. Clinical effects included (1) tarry stools, anorexia, emaciation, and death in 6 to 12 weeks (except for 1 female which was moribund when killed terminally) of 8/8 [REDACTED] on 200 ppm, and (2) slight elevation of SGOT and total bilirubin in the high dose females at 4 weeks, and of SGOT in 2/4 females on 100 ppm at 13 weeks.

2. Histo-pathological changes were: (1) bile stasis in 7/8 livers on 200 ppm, with necrosis in 4/8 at this level (severe in 3 and focal in 1), and slight parenchymatous degeneration with focal lymphocytic infiltration in 2/8 livers on 100 ppm (neither of the 2 dogs showed abnormal chemistries); (2) degeneration of the [REDACTED] tubules of the kidney in 4/8 on 200 ppm, with vacuolation of the tubular epithelium in 1/4 males and 2/4 females on 100 ppm, 1/4 of each sex on 50 ppm, and 1/4 female controls; and (3) mild or focal pneumonitis in 4/8 on 100 and 2/8 on 50 ppm. The severe autolysis in 4 dogs on 200 ppm made it impossible to determine whether the renal tubular and hepatocellular necrosis seen in the other 4 were also present in them.

B. Three-Generation Rat Reproduction Study.

Methods.

Albino Sprague-Dawley rats divided into 5 groups, each consisting of 10 males and 20 females, were fed either 0, 10, 50, 100, or 200 ppm. DAC-3701 in Ground Peerless Laboratory Animal Diet from the age of 7 weeks. After at least 70 days of treatment they were mated (1 male to 2 females) for 15 days. Gestation, [REDACTED] parturition, milk residues of the metabolite in the stomach of the pups at 7 days, and lactation to weaning (at 21 days, with parents for the next generation being selected at 28 days) were monitored, with weights, litter sizes, survival after various periods, and subjective observations being recorded. The same procedure was carried out 2 more times using offspring as parental animals for the next generation. A second litter was produced with 1/2 the 200 ppm rats from the first generation (F₁b). Autopsies were performed as follows: (1) P₀ generation - after 120 days of treatment as a subacute toxicity study to be described later, (2) F₁ - gross autopsies on pups which were stillborn or died and on parents (of F₂ generation) which died or were killed after the young were weaned, (3) F₂ - gross autopsies or skeletal clearing on stillborn pups, and gross autopsies on 5/sex/group of parents (of F₃ generation) as for F₁ parents, (4) F₃ weanlings - gross and microscopic examination of 5/sex/group (except for F₃ 200 ppm group, which was autopsied at 5 to 8 days of age as none survived to the age of weaning).

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Effects.

Effects of treatment increased in severity with increasing dose and generation, with the no-effect level being 10 ppm. The following changes were observed: (1) increase in mortality, marked at 200 ppm but slight at 50 and 100, (2) reduction in the viability index (% alive at one time as opposed to another) at 200 and 100 ppm, (3) decrease in fertility of the F₁ and F₂ generations at 200 ppm and the F₂ at 100 also, (4) reduction in litter size involving all 3 generations at 200 and 100 ppm but the F₃ only at 50 ppm, (5) decrease in weight of offspring at 200 and 100 ppm, (6) weakness of the F₃ pups, thinning and roughening of the fur of the F₁'s and F₂'s, increase in irritability of the P₀, F₁ and F₂ generations, and delay in healing of skin lesions in all generations at 200 ppm, (7) slight to moderate deposition of pigment in Kupffer cells in the liver of 2 F₃ females on 200 ppm which died at 7 days and moderate renal tubular vacuolation^{also} in 1 of the 2, and (8) detectable residues of the metabolite in the milk curd in the stomach after 7 days at all treated levels.

C. Four-Month Subacute Rat Feeding Study.Methods.

Sprague-Dawley rats serving as the P₀ generation for the reproduction study just described were divided into groups of 10 males and 20 females receiving either 0, 10, 50, 100, or 200 ppm of the metabolite in the diet for a total of 120 days. Testing procedures during life included observation, weighing, food consumption, hematology (erythrocyte count, total and differential leukocyte count, hematocrit, and hemoglobin), blood chemistry (BUN, fasting glucose, serum alkaline phosphatase, and SGPT), and urinalysis. At the end of the experiment all rats were autopsied, organ weights were obtained, and the following organs were examined microscopically: adrenals, aorta, brain, eye, heart, stomach, small intestine, colon, cecum, kidneys, liver, lung, trachea, seminal vesicle, pancreas, pituitary, prostate, uterus, testes, ovaries, spleen, bone marrow, thyroid, salivary gland, bladder, nerve, muscle, optic nerve, and unusual lesions of any other organ.

Effects.

The only effects were (1) occasional soft stools at the 2 upper doses, and (2) minor changes limited to 200 ppm and consisting of irritability, slight and statistically insignificant depression of growth in the females, and slight leukocytosis and increase in liver weight in the males. No histopathologic changes attributable to treatment were demonstrable in any organ. Although there were apparently no more changes in the kidneys of the treated rats than in those of the controls, all 5 groups showed an unusually great amount of renal disease (chronic nephritis,

calculi, nephrocalcinosis, and even 4 tumors) for animals no older than 6 months (at the end of the study).

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II. Further Consideration of Rat Study 200-205 on Daconil.

The many studies on Daconil, the parent compound, have been reviewed by myself and others in previous memoranda. In addition to causing ~~gastro-esophageal~~ gastro-esophageal acanthosis in rats and hepatic damage in both rats and dogs at high doses, this fungicide induces undeniable tubular lesions in the kidneys of both species ranging from severe at high dietary levels (500 through 30,000 ppm) to moderate at lower doses (250 ppm in the rat) and slight at very low levels (down to 120 ppm in the dog and 4 ppm in the rat). I can personally attest to the presence of the renal lesions at lower doses in both species, having studied the microslides myself, and I have also seen photomicrographs and photographs of the gross specimens of the severe changes ^{in rats} at high doses. While the no-effect level for the dog has been found to be 60 ppm, the no-effect level for the rat is still in question. In study 200-205 in which Charles River C-D rats were fed doses ~~of 0, 4, 10, 20, 30, 40, or 60 ppm~~ ^{of 0, 4, 10, 20, 30, 40, or 60 ppm} for 2 years, vacuolation of the cytoplasm of the pars recta of the proximal tubules of the kidney first appeared at 3 months at all treated levels, but by 12 months it was not seen below 30 ppm. While a few males were affected, a predilection for females was evident. At the termination of the experiment after 2 years, the epithelium of the entire proximal tubule in the deep or juxta-medullary region of the cortex in a total of 6 treated females, 4 on 4 ppm, 1 on 40, and 1 on 60, was the site of vacuolation plus severe degeneration with cell membrane disruption and nuclear chromatolysis and pyknosis; 6 other rats (all female except 1 male on 40 ppm), 3 on 30, 2 on 40, and 1 on 60 ppm, showed vacuolation only. ~~The degenerative change is unquestionably necrosis when it occurs in the living animal, but it is also characteristic of post-mortem autolysis.~~ The degenerative change is unquestionably necrosis when it occurs in the living animal, but it is also characteristic of ~~post-mortem~~ post-mortem autolysis. It is important to determine whether this represents necrosis or autolysis, for, while vacuolation is not necessarily serious and is potentially reversible, necrosis means death, and death of any tissue, especially in a vital organ such as the kidney, is always serious, even though the tubules are capable of regeneration. Arguments in favor of autolysis, which were advanced by 2 pathologists representing Diamond Shamrock Chemical Company, Klaus Stemmer, M.D., Pathologist and Associate Professor of Environmental Medicine, Department of Environmental Health, College of Medicine, University of Cincinnati (see memo of conference of January 17, 1974), and Simon Koletsky, M.D., Professor of Pathology, Case Western Reserve University Medical School, Cleveland, Ohio (see memo of conference of November 7, 1974), are (1) the peculiar distribution lacking any relation to dose and (2) the lack of an inflammatory reaction and of tubular regeneration, 2 features which

are expected in true or ante-mortem necrosis. While I respect both Dr. Kóletsky and Dr. Stemmer as pathologists and acknowledge the validity of their objection, there are other factors, namely, (1) the absence not only of this change but of tubular vacuolation in any of the controls at any time interval, and (2) the normal appearance of [REDACTED] other contiguous portions of the tubule and other elements of the surrounding parenchyma (especially in the subcapsular zone, which is ordinarily the first to undergo artefactual change due to drying), which point rather to some type of relation to the compound fed. I have been supported in this opinion [REDACTED] by 2 other EPA pathologists, Howard L. Richardson, M.D., and Mary E. Richardson, M.D. Delay in putting some of the kidneys showing vacuolation limited to the area in question into fixative, resulting in autolysis of the affected areas only rather than the kidney as a whole, [REDACTED] may explain the necrotic appearance, as abnormal tissue tends to autolyze earlier than intact. If such vacuolation, apparently an osmotic effect, is the only change at 4 to 60 ppm, it is unlikely that it would pose any real problem to man. However, because of (1) the severe osmotic nephrosis at higher levels [REDACTED] (characterized grossly by marked enlargement, granularity, and greenish-brown discoloration of the kidneys, and microscopically by hypertrophy, dilatation, intraluminal cast formation, and [REDACTED] vacuolation, ^{of the cytoplasm,} enlargement, pigmentation, and hyperplasia with stratification of the epithelial cells of the proximal tubules) associated with increased mortality, and (2) the inability to predict the human response with absolute accuracy, to be on the safe side, another attempt should be made to find the true no-effect level in the rat. One more study, properly done, might not only establish this but also elucidate the nature of the changes from 4 to 60 ppm. While at one time I thought another 2-year study, which the company is naturally reluctant to do, might be necessary, I am now of the opinion that one of only 3 or 4 [REDACTED] months should be sufficient, as vacuoles appeared at the lowest dose after this period. Levels from 4 ppm down to 1 ppm (and up to 60 ppm, also, if desired) should be used. The Charles River C₃D strain, which was used in previous experiments, should probably be used again; if not, some other strain with a low spontaneous incidence of renal disease should be selected. AS will be discussed later, a nutritionally adequate diet is also important. As the only effect at 4 to 60 ppm in experiment 200-205 was on the kidneys, the only procedure necessary would be examination of the kidneys, including microscopic study of every animal. Care should be taken to put the kidneys into formalin or other fixative as soon as they are removed from the body; [REDACTED] weighing of organs, the source of much artefactual drying,

is unnecessary for a special study such as this. It will be unfortunate if an effect should be present even at 1 ppm, However, if mild vacuolation is the only change this might not preclude granting the tolerances now being sought, assuming, of course, that the 4-hydroxy metabolite proves safe when testing is finally complete.

001098

DISCUSSION OF 4-HYDROXY METABOLITE OF DACONIL

Though almost all the Daconil fed to the rat and dog is supposed to be excreted unchanged, with only a small percentage being converted into metabolites (chiefly the 4-hydroxy metabolite), it is interesting that the effects of this 4-hydroxy metabolite or DAC-3701 in the dog, i.e., hepatic biliary stasis and hepatocellular and renal tubular necrosis at 200 ppm, with some hepatic damage at 100, and slight renal tubular vacuolation in the males at 100 and 50 ppm, are similar to those of the parent compound. (Although some of the females showed similar vacuolation, the presence of this change in 1/4 female controls and the known propensity for female dogs to develop it make it difficult if not impossible to evaluate in this sex.)

~~Since the effect of the 4-hydroxy metabolite in the dog is similar to that of the parent compound, it is possible that the effect of the parent compound in the dog is also similar to that of the 4-hydroxy metabolite.~~

Though the change at the lowest dose in the males was slight, a true no-effect level for the metabolite was not demonstrated in the dog.

Unlike the dog study, the 4-month rat feeding study on the 4-hydroxy metabolite at doses from 10 to 200 ppm did not show apparent histopathologic effects. However, because of the unfortunately high spontaneous incidence and severity of various types of renal disease (chronic nephritis, nephrolithiasis, nephrocalcinosis, and even tumors) in all groups, including the controls, it is difficult to be certain that the compound was not responsible for some of it. It is unusual to find so much in rats so young (they were only 6 months old at the end of the study); even though the strain used, the Sprague-Dawley, is known to develop chronic nephritis earlier than certain other strains. The presence of pelvic stones and intra-renal calcification suggests that a dietary deficiency, possibly of magnesium, which is known to cause this, might also have played a role. As the Osborne-Mendel rats used during my years with the FDA which were fed Purina Chow remained virtually free of disease until the age of approximately 1½ years while those on a synthetic diet developed early nephrocalcinosis, it is possible that the diet used, Ground Peerless Laboratory Animal Diet, was nutritionally inadequate. Diamond Shamrock is supposedly planning a 2-year rat study on this metabolite. I hope this will show a clear-cut no-effect level. However, if the animals involved

react similarly to those in the 4-month subacute study, they will all be dead long before the experiment is due to be terminated. If this study has not yet been started, it might be wise to use a strain less prone to develop early renal disease than the Sprague-Dawley, such as the Charles River or Osborne-Mendel, and to be sure the diet is nutritionally adequate.

SUMMARY AND RECOMMENDATIONS

Studies on DAC-3701 or 4-hydroxy-2,5,6-trichloroisophthalonitrile, the major metabolite of the fungicide Daconil, were requested because the tolerances now being sought will result in 0.14 ppm of this metabolite in milk. The chronic feeding study in rats has not yet been completed, but the 3-month dog feeding, 4-month rat feeding, and 3-generation rat reproduction studies have been done.

In a 3-generation reproduction study in which Sprague-Dawley rats were fed the 4-hydroxy metabolite at levels of 0, 10, 50, 100, or 200 ppm, effects of treatment increased in severity with increasing dose and generation, with the no-effect level being 10 ppm. Changes consisted of (1) increased mortality, (2) decreased fertility, litter size, and weight of offspring, and (3) effects limited to 200 ppm, including increased irritability, thinning and roughening of fur, poor healing of skin lesions, weakness of the F₃ generation, and renal tubular vacuolation in one F₃ female and increased intrahepatic pigment in 2. Residues of the metabolite were detectable in the milk curd in the stomachs of the offspring at 7 days of age in all generations and at all levels of treatment.

Though the only effects attributed to dietary administration of the metabolite to Sprague-Dawley rats at levels of 0, 10, 50, 100, and 200 ppm for 4 months were minor ones limited to the highest dose, the high incidence and severity of spontaneous renal disease in the controls made any ^{possible} effect of treatment on the kidney difficult [REDACTED] to demonstrate.

Feeding of 0, 50, 100, or 200 ppm of DAC-3701 in the diet to beagles, 8 per group, for 3 months produced dose-related effects varying from very slight at 50 ppm to severe at 200 ppm, a true-no-effect level not being demonstrated. Changes consisted of (1) emaciation followed by death in all on 200 ppm, (2) biliary stasis in 7/8 livers with necrosis also in 4/8 at 200 ppm, and slight hepatocellular degeneration with focal lymphocytic infiltration in 2/8 at 100 ppm, (3) renal tubular degeneration in 4/8 at 200 ppm, and tubular vacuolation in 1/4 males at both 100 and 50 ppm, and (4) pneumonitis in 4/8 on ~~100~~ 100 ppm and 2/8 on 50 ppm.

Despite many studies, which have been reviewed in previous memoranda, the no-effect level in the rat for the parent compound, Daconil, has still not been established because of the inability of Diamond Shamrock Chemical Company to satisfactorily explain certain alterations, i.e., tubular vacuolation with superimposed changes which may be either ~~the~~ true ante-mortem necrosis or post-mortem autolysis, in some of the kidneys at the lowest dose used, 4 ppm, as well as at higher doses up to 60 ppm in experiment 200-205. Necrosis of any tissue, especially a vital organ such as the kidney, is always serious. Furthermore, while mild tubular vacuolation ~~alone~~ alone, apparently an ~~osmotic~~ osmotic effect, would probably pose no real threat to man at these levels, because of (1) the severe osmotic nephrosis at higher levels in both dog and rat, and (2) the inability to predict the human response with absolute certainty, another attempt should be made to find the no-effect level in the rat. One more properly done study, which I requested in my memorandum of February 28, 1974, might both establish this level and explain the apparent necrosis at 4 to 60 ppm. However, although I originally thought another 2-year experiment would be necessary, I now believe one of only 3 or 4 months should be sufficient.

In view of the above, I should like to make the following recommendations.

1. A new 3-to 4-month feeding study with Daconil should be done in the rat, preferably the Charles River C-D, as this was used in previous studies, in an attempt to establish the type of renal changes present at very low levels and to determine the true no-effect level. Doses from 4 ppm down to 1 ppm should be used, with some up to 60 ppm, also, if desired. Laboratory tests will not be necessary. As there is no indication that other organs are affected at these low levels, only the kidneys need be examined, but microscopic study of these organs from every rat is essential. To ensure that drying artefacts or autolysis do not occur, there should be absolutely no delay in getting kidneys into formalin (or other fixative) after removal from the body.

2. In view of the high spontaneous incidence of moderate to severe renal disease in the controls in the 4-month study on the 4-hydroxy metabolite of Daconil in the Sprague-Dawley rat, I should like to suggest that, if the 2-year rat feeding study on this metabolite has not already been started, that a strain with less spontaneous renal disease be used, and also that the diet be checked for nutritional adequacy as well as for the possible presence of toxic agents. Consideration might be given to using Purina Rat Chow, which has given excellent results in my experience.

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