Protein-Deficient Diet and Diuron Toxicity

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The clinicopathologic syndrome of toxicity to diuron or 3-(3,4-dichlorophenyl)-1,1-dimethylurea, at the acute oral LD_{50}, was determined in male albino rats fed from weaning on diets containing 3.5% protein as casein (I), 26% casein (II), or laboratory chow (III). The $LD_{50} \pm$ S.E. was 437 $\pm$ 139, 2390 $\pm$ 1440, and 1017 $\pm$ 222 mg per kg in I, II, and III, respectively. The syndrome of toxicity at the LD_{50} was essentially the same in animals of all three dietary groups and consisted of signs of depression of the central nervous system, cholinergic stimulation, a local gastroenteritis, hepatitis, nephritis, dehydration, and loss of weight in body organs. Comparison of the results with corresponding data on other pesticides revealed that some are relatively much more toxic than others in the protein-deficient rats.

D iuron or 3-(3,4-dichlorophenyl)-1,1-dimethylurea is a general herbicide used selectively against certain weeds at 0.6 to 4.8 pounds per acre and nonselectively at 16 pounds per acre and over (Spencer, 1968). The substituted urea (chemical structure shown in Figure 1) is a white, odorless, crystalline powder which is insoluble in water and partially soluble in cottonseed oil. It is broken down in soil by removal of one and then two of the methyl groups on the urea moiety and by hydrolysis to 3,4-dichloroaniline, apparently by the action of soil organisms (Smith and Sheets, 1967). Diuron is readily absorbed by plant roots, translocated acropetally in the transpiration stream, and is in part demethylated. It is used for weed control in soil and water (Katz, 1966), and its presence in water may inhibit growth of food-chain microfauna, such as Daphnia, and interfere with the growth of aquatic animals such as fish (Crosby and Tucker, 1966).

Diuron is readily absorbed when given by mouth to mammals such as rats, is not appreciably stored in the body, and part of it is excreted unchanged in feces and urine (Hodge et al., 1967). In mammals it is metabolized by dealkylation of the urea methyl groups, by hydroxylation at carbons 2 and/or 6 of the benzene ring (Böhme and Ernst, 1965), by hydrolysis to 3,4-dichloroaniline, and by oxidation to 3,4-dichlorophenol (Hodge et al., 1967). A residue tolerance of 1 ppm for certain fruits and vegetables has been established in Canada (Chapman, 1967).

The basic objective of this study was to obtain information pertinent to the safety of diuron in countries where the diet is low in protein. Results on other pesticides (Boyd, 1969) indicated that, while lowering protein intake to one-third of normal had little effect, a lowering to one-seventh of the normal markedly increased the susceptibility of rats to the toxic effects of some pesticides, such as parathion and captan, but not appreciably to others, such as chlordane and lindane. As might be expected, when weaning rats are fed a diet containing no protein (on which they develop signs of kwashiorkor and lose body weight), susceptibility to the acute toxic effects of all pesticides is markedly increased but again to some much more than to others.

METHODS

The experiments were performed upon albino rats of a Wistar strain obtained from Woodlyn Farms Limited of

Guelph, Ontario. Group I consisted of 95 animals purchased as weanlings, weighing 50 to 60 g. They were fed for 28 days on a protein-deficient diet obtained as Protein Test Diet–Low from General Biochemicals of Chagrin Falls, Ohio. This diet was prepared after the formula of Hegsted and Chang (1965), and contained 3.5% casein, 81.5% cornstarch, 8% hydrogenated cottonseed oil, 4% salt mix, and 3% of an all vitamin mixture. The feeding period of 28 days was selected from the studies of De Castro and Boyd (1968), who found that weanling rats require 1 to 2 weeks to adapt to a protein-deficient diet. At the end of 28 days, the mean $\pm$ standard deviation body weight of animals in group I was 58 $\pm$ 8 g. They were then used for determination of the clinicopathologic syndrome of acute oral toxicity to diuron as described below.

Group II was a control group of 126 weanlings fed Protein Test Diet–Normal, also obtained from General Biochemicals and containing normal amounts (26%) of casein with 59% of cornstarch and other ingredients as in Protein Test Diet–Low. At the end of 28 days on this diet, they weighed 202 $\pm$ 15 g. Group III was a group of 88 albino rats purchased at 2 weeks after weaning and fed a standard laboratory chow for 2 weeks. The standard chow was Rockland Rat Diet Complete, obtained from Teklad Incorporated of Winfield, Iowa. It contained 25% of protein as mixed animal and plant proteins. At the end of the feeding period the animals weighed 172 $\pm$ 10 g.

The acute oral toxicity of diuron was then measured in animals of each of the three dietary groups. To empty the stomach, each animal was placed singly in a metabolism cage with water but no food for 16 hr (overnight). Diuron was used as Technical Diuron (95%) (Industrial and Biochemicals Department, E. I. du Pont de Nemours, Wilmington, Del.). It was freshly dissolved or suspended in cottonseed oil, U.S.P. XVII, and administered into the stomach through a cannula attached to a syringe. The volume was maintained at 20 ml per kg body weight because of evidence, reviewed by Boyd (1968), that variation in volume affects toxicity. The LD_{50} of diuron in each dietary group was approximated from pilot tests, and the herbicide was then given in a series of definitive doses estimated to produce mortality rates of from just above 0% to just below 100%, each dose to 10 rats of each dietary group with 15 controls of each dietary group given 20 ml per kg of cottonseed oil. The animal was then returned to its metabolism cage with a weighed excess of food, and a measured excess of drinking water for cageside observations of clinical signs of toxicity.

Clinical signs were quantitated in clinical units of 1+ to 4+

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Table I. The Interval to Death, Lethal Doses, Immediate Cause of Death, and Gross Pathology from Oral Administration of Diuron to Albino Rats

Measurement	Group I Previously Fed Protein Test Diet–Low	Group II Previously Fed Protein Test Diet–Normal	Group III Previously Fed Laboratory Chow
Hours to death: mean ± S.D.	23 ± 10	28 ± 13	24 ± 9
Estimated LD_1 (mg/kg)	131	392	328
LD_{50} ± S.E. (mg/kg)	437 ± 139[a,b]	2,390 ± 1,440[b]	1,017 ± 222
Estimated LD_{99} (mg/kg)	742[a,b]	4,380[b]	1,690
Gross Pathology[c]	Gastritis Enteritis Dehydrated cecum	Gastritis Enteritis Dehydrated cecum Congested brain Congested lungs Yellowish kidney	Gastritis Enteritis Dehydrated cecum Congested brain Congested lungs Yellowish kidney

[a] Significantly different from results in rats previously fed Protein Test Diet–Normal (group II) at $P = 0.02$ or less. [b] Significantly different from results in rats of group III previously fed Laboratory Chow at $P = 0.02$ or less. [c] The immediate cause of death was respiratory failure in each group.

at hourly intervals during the balance of the first day after administration of diuron, and then at intervals of 24 hr or as indicated, for 5 days. Survivors were returned to the animal boarding cages and observed for 1 month. At intervals of 24 hr for 5 days after herbicide administration, change in body weight was measured as g per 24 hr, food intake in g per kg body weight per 24 hr, water intake in ml per kg per 24 hr, colonic temperature in °F, urinary volume in ml per kg per 24 hr, urinary blood in units per kg per 24 hr, urinary glucose and protein output in mg per kg per 24 hr, and urinary pH on 24 hr specimens. Details of method have been reviewed by Boyd (1968).

Each animal which died was autopsied and gross pathology recorded. Histopathology was noted on any tissue which appeared abnormal to gross examination. When autopsy could be performed within an hour of death, to avoid postmortem changes described by Boyd and Knight (1963), histopathology and organ weights and water content were recorded upon the organs listed in Table II in a representative number of animals of each dietary group. Microscopic examinations were made upon blocks of tissue fixed in Lillie's buffered formalin, with sections stained by hematoxylin-phloxine-saffron.

The results were analyzed statistically by application of t tests of significance to differences between means and by analyses of the regressions of differences on dose of diuron or interval after administration of diuron. The LD_{50} ± S.E. was calculated by analysis of the linear regression of dose on mortality. Statistical methods have been reviewed by Boyd (1968).

RESULTS

Data on lethal doses are assembled in Table I. The interval to death varied inversely with the dose of diuron. The mean interval to death was about 24 hr after diuron administration in all three dietary groups. The LD_{50} ± S.E. in
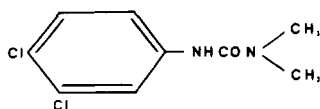
DIURON

Figure 1. The structural formula of diuron

group II was significantly higher than that in group III at $P < 0.02$, and both were significantly greater than that in group I. By this criterion, diuron was some five times more toxic to the protein-deficient animals than to those fed adequate amounts of protein as casein. When adequate amounts of protein were provided as casein, the rat was somewhat more resistant to diuron toxicity than when provided as the mixed protein of laboratory chow.

The clinical signs of intoxication were identical in rats of all three dietary groups. Within 2 hr of administration of diuron, there appeared drowsiness, hyporeflexia, and ataxia. The intensity of these signs increased during the subsequent 6 hr of the day of diuron administration. At 24 hr, animals which eventually died were prostrate and had bradypnea. Animals which subsequently survived exhibited irritability and hyperreflexia. At 24 hr there also appeared diarrhea, hemodacryorrhea, and epistaxis. Measurements at 24 hr disclosed a significant ($P < 0.01$) and marked hypothermia, oligodipsia, anorexia, loss of body weight, diuresis, glycosuria, proteinuria, and aciduria. The intensity of all of these signs was dose-dependent and, with the exception of irritability and hyperreflexia, was markedly increased just before death. The immediate cause of death was respiratory failure, as noted in Table I.

Gross examination of body organs at death revealed a local gastroenteritis with a marked drying of the contents of the cecum. The brain and lungs were congested and the kidneys appeared as yellowish grey in group II and III, as noted in Table 1.

Histopathological findings at death are summarized in Table II. Diuron produced capillary-venous congestion of the lamina propria and submucosa of the gastrointestinal tract, with some gastric ulcers and a stress reaction in the adrenal and thymus glands and spleen. There were toxic changes in the liver and kidneys. Protein-deficient rats exhibited, in addition, signs of kwashiorkor, such as inhibition of development of the adrenal and thymus glands, the gastrointestinal tract, and especially the testes.

Diuron produced loss of weight in most body organs, the loss being particularly marked in the intestinal tissues and spleen. Part of the loss in weight was due to dehydration, but the adrenal glands and occasionally the salivary glands were hydrated.

Signs of recovery began to appear in survivors at 24 hr and were quite evident at 48 hr. By 72 hr, most of the clinical signs of toxicity had disappeared. Body temperature and

Table II. Histopathological Changes in Albino Rats at Death from Oral Administration of Diuron

Organ	Histopathology
Adrenal glands	Lipoid globules prominent in *zona fascicu-lata;* undeveloped in protein-deficient rats
Brain	Hyperemic with occasional small capillary hemorrhages
Gastrointestinal tract:	
Cardiac stomach	Mild congestion of the submucosa
Pyloric stomach	Capillary-venous congestion of the lamina propria adjacent to the mouths of the gastric glands with occasional ulcer formation
Small bowel	Congestion of the intestinal villi with un-developed villi in protein-deficient rats
Cecum	Capillary-venous congestion and shrunken Lieberkuhn glands
Colon	Normal appearance
Heart	Normal appearance
Kidneys	Congestion of the glomerulus and tubules, especially in the region of the loop of Henle; tubular cloudy swelling and fatty degeneration
Liver	Centrilobular cloudy swelling, fatty de-generation and early necrosis
Lungs	Parenchymal capillary-venous congestion
Muscle (ventral abdominal wall)	Normal appearance
Pancreas	Islet tissue ischemic; deficiency of zymo-genic granules in the acinar glands
Salivary (sub-maxillary) glands	Normal appearance
Skin	Ischemic; epidermis thin in protein-de-ficient rats
Spleen	Splenic corpuscles edematous; red pulp congested; phagocytized debris; shrunken
Testes	Normal appearance except in protein-de-ficient rats which exhibited no sperma-togenesis beyond the spermatocyte stage
Thymus gland	Loss of thymocytes especially in protein-deficient rats

water intake had returned to normal at 48 hr and growth rate at 72 hr, in spite of some residual anorexia. The glycos-uria, proteinuria, and aciduria had practically disappeared at 72 hr, and recovery was accompanied by a diuresis.

DISCUSSION

The results of this study demonstrated that albino rats fed for 28 days from weaning on a diet containing one-seventh the optimal intake of protein are highly susceptible to the toxic effects of diuron herbicide. The clinicopathologic syndrome of toxicity from doses at the range of the oral LD_{50} was essentially the same in these animals as in animals fed adequate amounts of protein as casein or as the mixed pro-tein of laboratory chow.

Data on diuron have been compared in Table III, with cor-responding available data cited by Boyd and Taylor (1970) on 15 other pesticides in the form of an assemblage of values for the acute oral LD_{50} in animals fed for 28 days from weaning on a protein deficient diet. These values have been related to values in animals previously fed a diet containing normal amounts of protein as casein and as the mixed pro-teins of laboratory chow. It will be noted that animals fed for 28 days from weaning on Protein Test Diet–Low were more susceptible to the acute oral toxicity of all pesticides except dimethoate. To obtain an index of the augmented susceptibility, the LD_{50} in rats fed Protein Test Diet–Normal was divided by the LD_{50} in rats fed Protein Test Diet–Low and the resulting figures are shown in parentheses under the column headed "Protein deficient (3.5% casein) diet" in Table III.

Chlordane, diazinon, endrin, lindane, and malathion were up to twice as toxic in animals previously fed a diet containing 3.5% casein as in animals fed 26% of dietary casein. Seven other pesticides were 3 to 5 times more toxic in animals fed the protein-deficient diet than in animals fed a normal casein diet, namely chlorpropham, dicophane or DDT, diuron, endosulfan, monuron, toxaphene, and demeton.

Carbaryl is a naphthylcarbamate and was six times more toxic in the protein-deficient rat. Parathion is a *para*-nitrophenyl organic phosphorothioate and was eight times

Table III. The Acute Oral LD₅₀ of Sixteen Pesticides in Male Albino Rats Fed for 28 Days from Weaning on a Diet Deficient in Protein Compared with Values in Controls Fed Normal Amounts of Protein as Casein or as Laboratory Chow[a]

Pesticide	Protein-Deficient (3.5% Casein) Diet Group I[b]	Normal Protein (26% Casein) Diet Group II[c]	Laboratory Chow Diet Group III
Captan	480 ± 110 (26)	12600 ± 2100 (1.0)	12500 ± 3500
Carbaryl	89 ± 11 (6)	575 ± 51 (1.3)	744 ± 40
Chlordane	137 ± 30 (2)	267 ± 44 (1.2)	311 ± 44
Chlorpropham	2590 ± 480 (4)	10390 ± 1580 (0.4)	4440 ± 480
Demeton	2.13 ± 0.37 (4)	7.62 ± 0.22 (1.4)	10.40 ± 0.77
Diazinon	215 ± 26 (2)	415 ± 39 (1.1)	466 ± 87
Dicophane (DDT)	165 ± 34 (3)	481 ± 13 (0.8)	368 ± 38
Dimethoate	147 ± 29 (1)	152 ± 22 (2.4)	358 ± 9
Diuron	437 ± 139 (5)	2390 ± 1440 (0.4)	1017 ± 222
Endosulfan	24 ± 10 (4)	102 ± 16 (1.2)	121 ± 16
Endrin	6.69 ± 0.80 (2)	16.60 ± 3.01 (1.6)	27.24 ± 6.54
Lindane	95 ± 33 (2)	184 ± 16 (0.9)	157 ± 37
Malathion	599 ± 138 (2)	1401 ± 99 (0.8)	1090 ± 83
Monuron	950 ± 240 (3)	2880 ± 310 (0.5)	1480 ± 310
Parathion	4.86 ± 1.31 (8)	37.1 ± 4.9 (0.6)	23.4 ± 5.4
Toxaphene	80 ± 19 (4)	293 ± 31 (0.8)	220 ± 33

[a] The LD₅₀ is expressed as mg/kg ± standard error; the data are from publications cited by Boyd and Taylor (1970). [b] In parenthesis is the value obtained by dividing the LD₅₀ in group II by that in group I. [c] In parenthesis is the value obtained by dividing the LD₅₀ in group III by that in group II.

more toxic. Captan is a mercaptophthalidimide related structurally to thalidomide, and was 26 times more toxic.

Of the 13 pesticides which were not over five times more toxic to the protein-deficient rat, nine were organochloro compounds and four were organic phosphorothioates. Of the three pesticides which were over five times more toxic to the protein-deficient rat, two (carbaryl and captan) had a quite different chemical structure and one (parathion) was an organic phosphorothioate but with a *para*-nitrophenyl group. Considered in this context, organic chloro pesticides of the DDT-lindane-chlordane type and organic phosphorothioates except parathion would appear the least likely to produce augmented acute oral toxicity in countries where the diet is low in protein. Carbaryl, parathion, and particularly captan would appear to be most likely to produce augmented toxicity reactions.

The above calculations of augmentation of susceptibility of protein-deficient rats to pesticides were based upon differences between animals fed from weaning on a diet containing 3.5% of protein as casein and controls previously fed normal amounts of dietary protein as 26% casein. If results in animals previously fed laboratory chow are used as controls, slightly different augmentations of toxicity are obtained. For example, chlorpropham becomes twice rather than four times more toxic in the protein-deficient rat, whereas endrin becomes four times rather than twice as toxic. While several calculations of augmented susceptibility to pesticides using

animals of group III as controls are significantly different statistically from calculations based upon group II controls, the differences are in general not great. With group III controls, pesticides more than five times as toxic in the protein-deficient rat are captan, carbaryl, demeton, endosulfan, and parathion.

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