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Interaction between γ-Hexachlorocyclohexane and the Gastrointestinal Microflora and Their Effect on the Absorption, Biotransformation, and Excretion of Parathion by the Rat

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Pretreatment of rats with the organochlorine insecticide lindane reduced the estimated absorption rate of parathion from the gastrointestinal tract. Lindane pretreatment also significantly reduced the metabolism of parathion to *p*-nitrophenol in vivo. Lindane pretreatment altered the gastrointestinal (GI) microflora by increasing the ratio of anaerobes to aerobes. Consistent with this alteration was a significantly greater retention of unaltered parathion and the microbial metabolite aminoparathion in the GI tract of the lindane-pretreated rats 1 h after the administration of parathion. Enhanced conversion of parathion to aminoparathion together with a slower absorption rate may play a role in the antagonism parathion toxicity by lindane.

In the past it has been widely reported that pretreatment of animals with halogenated chemicals antagonizes the toxicity of organophosphate pesticides (Ball et al., 1954; Triolo and Coon, 1966; Townsend and Carlson, 1981; Iverson, 1976; Mendoza and Shields, 1976; Welch and Coon, 1964; Triolo et al., 1970). Moreover, this antagonism has generally been attributed to induction of either esterases or hepatic mixed function oxidases. While examining the dynamics of the absorption, biotransformation, and excretion of parathion [*O,O*-diethyl *O*-(*p*-nitrophenyl) phosphorothioate], we observed that pretreatment of rats with lindane (γ-hexachlorocyclohexane) impaired the estimated absorption rate of parathion. Corresponding to this impaired absorption, significantly lower excretion rates during the initial absorption were also observed in the lindane-pretreated rats. A series of experiments designed to determine the mechanism by which lindane elicited these effects produced data that indicate that altered gastrointestinal microflora may contribute to the protective effect of lindane and possibly other halogenated chemicals against the toxicity of ingested organophosphate pesticides.

MATERIALS AND METHODS

Apparatus. Gas–liquid chromatographic analysis was performed on a Tracor Model MT-220 gas chromatograph equipped with a ^{63}Ni electron capture detector (ECD) and a flame photometric detector (FPD). Urinary *p*-nitrophenol was determined by ECD on 1% SP1240-DA on 100–120-mesh Supelcoport at 170 °C with 60 cm^3/min of 95:5 methane–argon carrier gas. Derivatized diethyl phosphorothioic acid (DETP), diethylphosphoric acid (DEP), and paraoxon [*O,O*-diethyl *O*-(*p*-nitrophenyl) phosphate] were determined by FPD with 3% OV-1 on 80–100-mesh Chromosorb W at 210 °C. Aminoparathion and parathion were analyzed by FPD with 3% QF-1 on 80–100-mesh Gas-Chrom Q at 185 °C. Air, H_2, and N_2 carrier gas flows were regulated at 100, 50, and 60 cm^3/min, respectively.

Reagents. [*ring*-2,6-^{14}C]Parathion (specific activity 12.2 mCi/mmol and 98% purity) was obtained from Amersham Corp., Arlington Heights, IL. Parathion, aminoparathion, DETP, DEP, paraoxon, and lindane were obtained from the EPA, Health Effects Research Laboratory Analytical Reference Standards Repository, Research Triangle Park, NC. Pentafluorobenzyl bromide (PFB-Br) was obtained from Aldrich Chemical Co., Milwaukee, WI. Tetrahexylammonium hydrogen sulfate was obtained from Regis Chemical Co., Morton Grove, IL; 3% QF-1 on 80–100-mesh Gas-Chrom Q and 3% OV-1 on 80–100-mesh Chromosorb W were obtained from Applied Science Laboratories, Inc., State College, PA. 1% SP-1240 DA on 100–120-mesh Supelcoport was obtained from Supelco Inc., Bellefonte, PA. Thioglycollate medium was obtained from Becton, Dickinson and Co., Cockeysville, MD. Bacto nutrient broth was obtained from Difco Laboratories, Detroit, MI.

Procedures. Separate experiments were conducted to determine (1) the effects of pretreatment with lindane on

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the absorption and excretion of parathion, (2) the effect of lindane pretreatment on the in vivo metabolism of parathion, (3) the effect of lindane pretreatment on the GI microflora, and (4) the effect of lindane-altered microflora on the disposition of parathion in the GI tract 1 h after administration. Throughout this study all insecticides were administered in a volume of 0.1% of the body weight of the treated animal. Controls received the same volume of the unadulterated vehicle.

To determine the effect of lindane pretreatment on the absorption and excretion of parathion, 36 weanling, female, Sprague-Dawley rats were randomly assigned to one of four treatment groups. Nine rats were pretreated po with 20 mg of lindane kg^{-1} day^{-1}, in peanut oil, for 14 days. The remaining rats were dosed for the same period with the peanut oil vehicle. On day 15, all rats received 0.325 mg/kg [*ring*-2,6-^{14}C]parathion in propylene glycol. The lindane-pretreated rats and another group of nine animals were dosed perorally with the [*ring*-2,6-^{14}C]parathion. Nine rats were dosed topically with an acetone–propylene glycol (1:1 v/v) solution of the [*ring*-2,6-^{14}C]parathion. The dosing solution was applied to a previously shaved area in the dorsal mid lumbar region of approximately 7.5 cm^2. The treated area was immediately covered by a perforated plastic blister from a Millex-SR 0.5 μm single-use filter unit, which was cemented to the animals' back with cyanoacrylate adhesive. Nine rats received an iv bolus dose of the propylene glycol solution of [*ring*-2,6-^{14}C]parathion. Urine, feces, and expired air were collected at 2-h intervals for 8 h and at 4-h intervals for 16 h. Total excreted ^{14}C radioactivity was used, in a procedure reported by Ramsey and Gehring (1981), to characterize the model, to obtain optimum estimates of the absorption and excretion rates of parathion, and to determine the effect of pretreatment with lindane. Urine was analyzed for the parathion metabolite DETP by the method of Bradway et al. (1981). Pharmacokinetic analysis was performed on the interval excretion data by using a nonlinear regression program NLIN (SAS Institute Statistical Analysis System, 1979). To derive the rate constant, K_e, controlling the excretion process from the rats dosed intravenously, eq 1 was used,

$$E_i = D_0 e^{-K_e t_i}(e^{K_e \Delta t} - 1) \quad (1)$$

where E_i is the interval amount of chemical excreted by this model during any time interval, D_0 is the total absorbed dose, t_i is the time at the end of the collection interval, and Δt is the duration of the collection interval. Though urinary DETP content was also used to test the validity of the model, some of the DETP data were missing and thus the rate constants reported in this study were estimated on the basis of the total excreted radioactivity. There were 72 experimental data points per treatment group used in fitting eq 1 to the data. To test if the single-compartment first-order excretion model was correct, the residuals of the difference between the observed and predicted values (from fitting the model to each of the individual rats) were tested by analysis of variance. Interval excretion data from rats dosed perorally or topically were also modeled by using the average excretion rate constant, K_e, estimated for the iv-dosed animals. Estimates of D_0 and the absorption constant, K_a, were obtained by fitting eq 2 to the data by using NLIN (SAS Institute Statistical Analysis System, 1979).

$$E_i = \frac{D_0}{K_a - K_e}[K_a e^{-K_e t_i}(e^{K_e \Delta t} - 1) - K_e e^{-K_a t_i}(e^{K_a \Delta t} - 1)] \quad (2)$$

To determine the effect of lindane pretreatment on the in vivo metabolism of parathion, six weanling, female, Sprague-Dawley rats were fed unadulterated Purina Lab Chow while six more were fed Purina Lab Chow containing 200 ppm of lindane for 3 months. Then all rats were dosed perorally with 1 mg of parathion/kg. Twenty-four-hour urine samples were then collected, extracted, and analyzed for DETP, DEP, and *p*-nitrophenol. DETP and DEP were derivatized and analyzed by FPD via a method reported by Reid and Watts (1981). An extractive alkylation procedure was employed to determine the urinary content of *p*-nitrophenol (Bradway et al., 1981) following acid hydrolysis of the urine. The *p*-nitrophenol was extracted from urine as an ion pair with the lipophilic quaternary ammonium cation from tetrahexylammonium hydrogen sulfate and alkylated with pentafluorobenzyl bromide.

To examine the effect of lindane pretreatment on the GI microflora, fecal samples, aseptically removed from the terminal portion of the descending colon, were collected from controls and rats fed 200 ppm of lindane in their diet for 1 month. Separate fecal samples from each rat were homogenized in thioglycollate and bacto nutrient broth and incubated for 24 h. The fecal samples in the thioglycollate medium were incubated under N_2 while those in bacto nutrient broth were incubated under air. Appropriate aliquots from both media were then counted on a hemacytometer at 450× magnification.

The effect of lindane-altered microflora on the disposition of parathion in the GI tract was determined 1 h after administration of 3 mg of parathion/kg to six control rats and six rats pretreated with 200 ppm of lindane in their diet for 1 month. The intestinal tract was removed and flushed with methanol–H_2O (1:1 v/v) to determine unabsorbed parathion and parathion metabolite content. The methanol–H_2O mixture was added to an equal portion of 1-propanol and subjected to azeotropic distillation at 60° in a rotary evaporator. After evaporation to dryness, ethyl acetate was added to the sample residue and this extract was analyzed by FPD.

Analysis of variance procedures and the Student's *t* test (Snedecor and Cochran, 1967) were employed as aids in the interpretation of the data from this study. Differences were considered significant at $p < 0.05$ unless otherwise stated.

Table I. Mean Residuals from Modeling Interval Excretion Data[a]

collection period[b]	intravenously dosed rats: radioactivity[c]	intravenously dosed rats: DETP[d]	rats not dosed intravenously: radioactivity[c]	rats not dosed intravenously: DETP[d]
0–2	9.24	2.08	5.61	1.32
2–4	−0.58	−0.11	2.48	−0.64
4–6	−5.46	−0.25	−0.86	0.30
6–8	2.98	−0.89	1.20	0.43
8–12	−0.79	0.56	3.02	−0.37
12–16	−1.33	0.10	−4.32	−1.34
16–20	0.00	−0.19	−2.09	−0.82
20–24	−0.28	0.19	−1.29	−0.14

[a] Values are means for nine rats. [b] Time, in hours, during which excreta samples were collected. [c] Values are percent of administered dose excreted. [d] Values are micrograms excreted.

RESULTS AND DISCUSSION

Effect of Lindane on Absorption and Excretion of Parathion. The residuals from fitting the single-compartment first-order excretion model (eq 1) to total radioactivity and urinary DETP from individual rats dosed iv with 0.325 mg/kg [*ring*-2,6-^{14}C]parathion are presented in Table I. These residuals were tested by analysis of variance to determine if the means varied significantly by collection period. There were no significant differences

Table II. Estimated Absorbed Dose (D_0), Excretion Rate Constants (K_e), and Absorption Rate Constants (K_a) for Parathion in the Rat[a]

treatment[b]	D_0, % administered dose	K_e, h^{-1}	K_a, h^{-1}
iv	100	0.18 ± 0.04	
po	102 ± 6.5	0.18	5.4 ± 1.5
dermal	87 ± 9.6	0.18	0.41 ± 0.12[c]
γ-HCH po	83 ± 8.1	0.18	0.88 ± 0.20[c]

[a] Values are means ± SE for nine rats. [b] Treatment is described under Materials and Methods. [c] Significantly different from the perorally treated rats at $p < 0.05$.

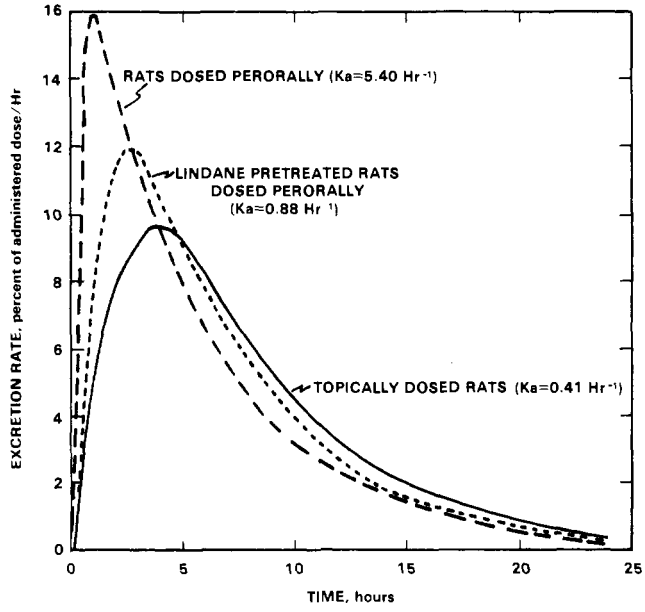

Figure 1. Effect of route of administration and pretreatment on the instantaneous excretion model of control rats dosed po with parathion (−−), lindane-pretreated rats dosed po with parathion (- - -), and rats dosed topically with parathion (—). There were nine rats per treatment, and collections were made at 2-, 4-, 6-, 8-, 12-, 16-, 20-, and 24-h intervals.

at $p < 0.05$, indicating that the model was adequate.

Excretion data from the 27 rats dosed by routes other than iv were also modeled (eq 2) and tested for lack of fit (Table I). Since residuals represent the difference between the observed data and that predicted by the model, it can be seen that the mode underestimates excretion at the 2-h collection and overestimates excretion at the 4-h collection. This suggests that going to a two-compartment model will not help since the two-compartment model predicts delayed excretion. Moreover, while the number of kinetically distinguishable compartments is theoretically unlimited, it is unlikely that interval excretion data will permit the resolution of more than two compartments (Ramsey and Gehring, 1981).

The estimated absorbed dose and absorption rate constants for all routes of administration are presented in Table II. The estimated absorption rate for the rats perorally dosed with parathion was 6 times greater than that for the lindane-pretreated rats dosed perorally with parathion and 13 times greater than that for rats dosed topically with parathion. Corresponding to these changes in the absorption rate were significant differences in the excretion rate during the initial absorption phase with both the topically dosed rats and the lindane-pretreated animals exhibiting lower excretion rates than the control rats perorally dosed with parathion (Figure 1). Figure 1 represents the derivative with respect to time from fitting eq 2 to the accumulative radioactivity excreted by the rats during successive time intervals. While a reduced initial excretion rate would be expected due to the relatively slower absorption of parathion through the skin, the decreased initial excretion rate observed in the lindane-pretreated rats was unexpected. Lindane induces hepatic mixed function oxidases (Kolmodin-Hedman et al., 1971; Chadwick et al., 1971; Den Tonkelaar and Van Esch, 1974; Mikol et al., 1980) and, if anything, might be expected to increase the excretion rate. Moreover, increases in the metabolism of paraoxon by mixed function oxidases have been cited as a mechanism by which halogenated compounds antagonize the toxicity of parathion (Townsend and Carlson, 1981; Iverson, 1976). Thus, the effect of lindane pretreatment on the metabolism of parathion was next examined.

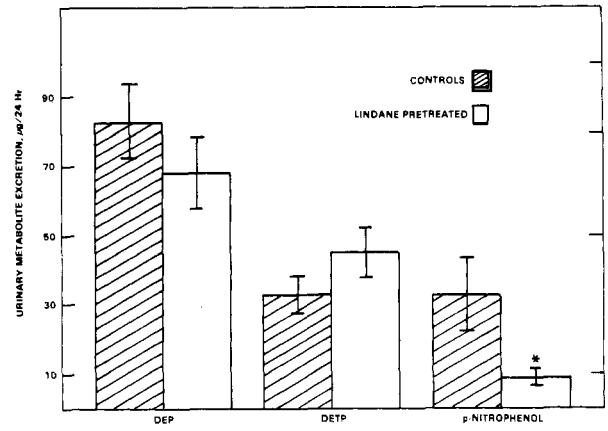

Figure 2. Effect of lindane pretreatment on the excretion of parathion metabolites in the urine. Shaded bars represent the controls while the open bars represent the lindane-pretreated rats. Each bar represents the mean of six rats. The vertical lines indicate the SE. The asterisk indicates a significant difference at $p < 0.05$.

Effect of Lindane on in Vivo Parathion Metabolism. The effect of lindane pretreatment on the metabolism of parathion is presented in Figure 2. The only significant difference observed was a marked reduction in the urinary excretion of *p*-nitrophenol in rats pretreated with lindane.

Since both parathion and paraoxon hydrolyze to DEP, DETP, and *p*-nitrophenol, this reduction in urinary *p*-nitrophenol was puzzling until the work of Mendel and Walton (1966) was recalled. These workers had reported that the normal flora from the gastrointestinal tract, and not the liver, were responsible for the conversion of DDT to DDD. Moreover, recent studies had demonstrated that soil microorganisms were active in the degradation of parathion (Adhya et al., 1981; Ferris and Lichtenstein, 1980). The possibility that microflora might be involved in the lindane-induced alteration of parathion metabolism was based on the following inferences: (1) rat GI microflora can readily reduce the nitro group (Wheeler et al., 1975); (2) reduction of the nitro group of parathion produces aminoparathion, which in turn hydrolyzes to DEP, DETP, and *p*-aminophenol (Ahmed et al., 1958); (3) since DETP and DEP arise from the hydrolysis of either parathion or aminoparathion, while *p*-nitrophenol is not produced when aminoparathion is hydrolyzed, an increase in the conversion of parathion to aminoparathion could account for the reduced level of *p*-nitrophenol excreted by lindane-pretreated animals. From these considerations, experiments were conducted to determine (1) whether lindane pretreatment affected the GI microflora and (2) whether microflora from lindane-pretreated rats altered the me-

Table III. Effect of Lindane Pretreatment on Fecal Microorganisms[a]

treatment[b]	aerobic microorganisms per g of feces × 10^{-10}	anaerobic microorganisms per g of feces × 10^{-10}	anaerobes/aerobes
control	1.27 ± 0.138	1.45 ± 0.212	1.13 ± 0.094
lindane	0.511 ± 0.020[c]	1.68 ± 0.071	3.32 ± 0.220[c]

[a] Values are means ± SE for four rats. [b] Treatment is described under Materials and Methods. [c] Significantly different from the controls at $p < 0.05$.

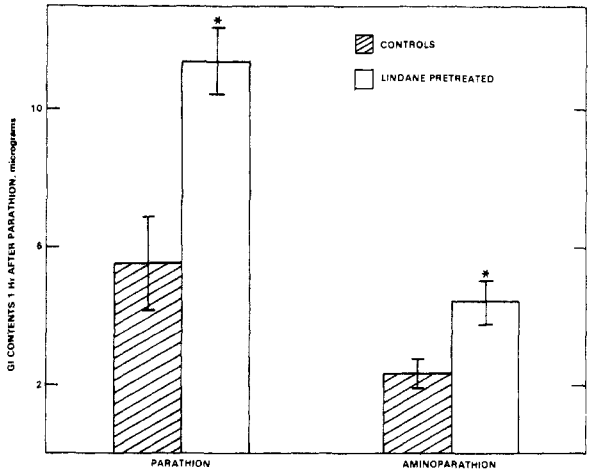

Figure 3. Effect of lindane-altered microflora on the disposition of parathion and the microbial metabolite, aminoparathion, 1 h after administration of parathion. The shaded bars represent the controls while the open bars represent the lindane-pretreated rats. Each bar represents the mean of six rats. The vertical lines indicate the SE. The asterisk indicates a significant difference at $p < 0.05$.

tabolism and disposition of parathion in the gastrointestinal tract.

Effect of Lindane on GI Microflora. Lindane pretreatment significantly decreased the number of aerobes and resulted in a 3-fold increase in the ratio of anaerobes to aerobes found in control feces under the conditions of this experiment (Table III). Assuming that fecal bacteria are representative of those in the GI tract, these data suggest that lindane pretreatment significantly altered the GI microflora in this experiment.

Lindane-Altered Microflora and Parathion Disposition. One hour after the peroral administration of parathion, it was observed that lindane pretreatment resulted in the retention of significantly higher quantities of both unaltered parathion and the microbial metabolite, aminoparathion (Figure 3). This represented 1.28 and 2.75% of the administered dose for control and pretreated rats, respectively. Since a material balance, employing exhaustive extraction of the walls of the GI tract or of the filtered food and feces, was not carried out, recoveries of unbound parathion and aminoparathion are probably low. However, under identical conditions, rats pretreated with lindane retained twice as much parathion and aminoparathion at this early time than did control animals. The higher level of parathion and aminoparathion in the GI tract of the lindane-pretreated rats, 1 h after administration, is consistent with the reduced absorption rate (Table II) as well as the apparent reduction in the excretion rate during the initial absorption phase (Figure 1). In addition, these data help to account for the lower level of *p*-nitrophenol excreted by the lindane-pretreated rats since elevated levels of parathion in the GI tract reflect impaired

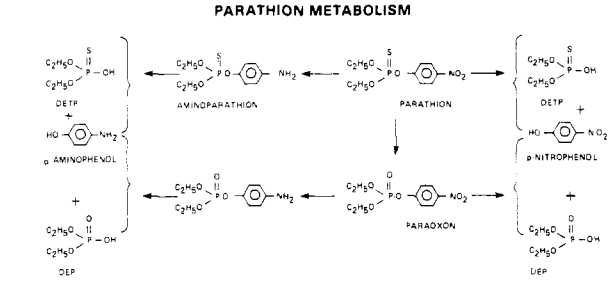

Figure 4. Parathion metabolism by ruminants (left) and nonruminants (right).

hydrolysis to *p*-nitrophenol via a reduced population of aerobes (Table III). Also, aminoparathion, whose level was increased in these rats, would be subsequently hydrolyzed to *p*-aminophenol instead of *p*-nitrophenol.

In the past it was reported that ruminants excrete *p*-aminophenol as a major parathion metabolite (Pankaskie et al., 1952) (Figure 4). This is believed to be the result of the ability of rumen organisms to reduce parathion to aminoparathion (Pankaskie et al., 1952; Ahmed et al., 1958; Cook, 1957). Soil microorganisms also degrade parathion and utilize both enzymatic hydrolysis and nitroreductase pathways (Adhya et al., 1981). Aerobic conditions promoted hydrolysis of parathion whereas anaerobic conditions promoted reduction of the nitro group. Moreover, the application of fungicides, herbicides, and N fertilizers produced an interaction with soil microorganisms and significantly affected their metabolism of parathion (Ferris and Lichtenstein, 1980). Soil treated with maneb, benomyl, glucose, or $(NH_4)_2SO_4$ exhibited extensive reduction of parathion together with impaired hydrolysis of the insecticide. Apparently, the application of these chemicals selectively inhibited only those soil microorganisms responsible for the hydrolysis and oxidation of parathion. Data from our study indicate that the normal flora in the gastrointestinal tract of the rat, like the rumen organisms and soil microflora, are able to degrade parathion. Pretreatment with lindane altered the microflora population by increasing the proportion of anaerobes at the expense of the aerobes (Table III). Thus, it appears that not only the number but also the composition of the microflora affect the fate of parathion and could account for the higher levels of both aminoparathion and parathion recovered from the GI tract of these rats. Since aminoparathion is 100-fold less toxic than parathion (Ahmed et al., 1958), enhanced conversion of parathion to this metabolite together with a reduced rate of absorption could help account for the antagonistic effect of lindane pretreatment on the toxicity of parathion (Ball et al., 1954). Previously, Ball et al. (1954) reported that aldrin pretreatment afforded the same degree of protection against both oral and intraperitoneal injections of parathion though they presented no data to support this claim. Triolo and Coon (1966), on the other hand, clearly demonstrated that the protection against parathion toxicity resulting from aldrin pretreatment was significantly greater when parathion was administered orally than when it was injected intravenously. Moreover, both Main (1956) and Triolo and Coon (1966) noted that aldrin protected rats against oral but not intravenously administered paraoxon. Since paraoxon can be reduced by microorganisms to aminoparaoxon, which is 300 times less toxic than paraoxon (Ahmed et al., 1958; Figure 4), aldrin-altered microflora may also have played a role in this protective effect. Thus, this work leads to speculation that altered GI microflora may also contribute to the protective effect of other hal-

ogenated chemicals against the toxicity of organophosphates.

Registry No. DEP, 598-02-7; DETP, 2465-65-8; lindane, 58-89-9; parathion, 56-38-2; *p*-nitrophenol, 100-02-7; aminoparathion, 3735-01-1.

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Fungicidal and Molluscicidal Activity of Some 3-Substituted 4-Hydroxycoumarin Derivatives

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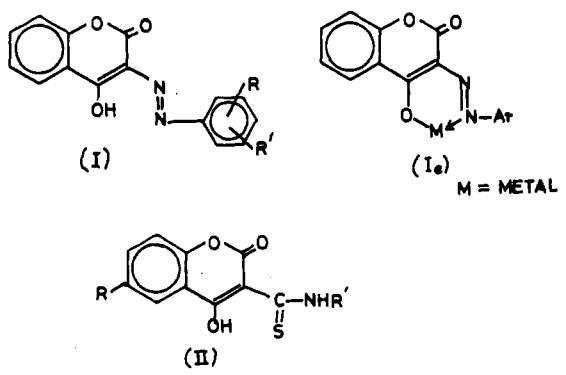
A number of derivatives comprising 3-(arylazo)-4-hydroxycoumarins (I) and 3-*N*-arylthiocarbamoyl)-4-hydroxycoumarins (II) have been synthesized. Eleven such compounds have been screened for their fungitoxicity against *Alternaria tenuis* and *Helminthosporium oryzae*, and two of them have been tested for their molluscicidal activity against *Lymnea acuminata*. The results have been compared with two commercial fungicides, Dithane M-45 and Bavistin, tested under similar conditions.

A number of coumarin and isocoumarin derivatives have been synthesized as insecticides (Beriger, 1976) and fungicides (Maikawa and Yoshikawa, 1979; Giri and Singh, 1978; Nakajima et al., 1979). The 4-hydroxyoumarin derivatives such as dicoumarol (O'Connor, 1948), warfarin (Overman et al., 1944), and coumachlor (Reiff and Weismann, 1951) are well-known rodenticidal agents. A number of azo compounds have been found to be active acaricides (Metcalf, 1955). Several azo dyes exhibit fungicidal activity (Silk and Summers, 1963). In view of these observations, it was anticipated that a compound of the structure I having a combination of an azo linkage with a 4-hydroxycoumarin moiety would possess interesting pesticidal properties. This is because the structure I would very well serve as a suitable ligand to chelate the essential metals involved in fungal metabolism as shown in Ie.

The importance of the N–C–S linkage as the active toxophore (Metcalf, 1955; Goldsworthy, 1942; Joshi and Giri, 1963) in a number of pesticides has been well stressed. 3-(*N*-Substituted carbamoyl)-4-hydroxycoumarins have been patented (Beriger, 1976) as an insecticide; it was expected that the analogous compounds (II) might also be active pesticidal agents.



The 3-(arylazo)-4-hydroxycoumarins (I) were prepared by reacting different aryldiazonium salts with 4-hydroxycoumarin in the presence of anhydrous CH_3COONa and

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