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PLANT METABOLISM OF INSECTICIDES

Identification of Metabolites of Zectran Insecticide in Broccoli

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The metabolism of Zectran in broccoli is reported. In addition to a small amount of Zectran, the following metabolites were found to be present in the broccoli flower: 4-dimethylamino-3,5-xylenol, 2,6-dimethylhydroquinone, 2,6-dimethyl-p-benzoquinone, and 4-dimethylamino-3,5-dimethyl-o-benzoquinone. The xylenol was found in both free and conjugated form, but the hydroquinone derivative was detected only as a water-soluble conjugate. Evidence is presented which suggests that metabolites of this pesticide have been incorporated into lignin.

ZECTRAN (registered trademark of The Dow Chemical Co.), 4-dimethylamino-3,5-xylyl methylcarbamate, is a highly active material for control of a large number of arthropod pests of plants (*5, 6, 8, 10, 12, 14*). It is toxic also to mollusks including some of the economically important plant-eating slugs and snails (*7*). Its potential use on food crops necessitated a study of its metabolism in plants.

Very little is known concerning the actual identity of carbamate degradation products in plants (*4*). The purpose of this investigation is to identify the metabolites formed when broccoli is treated with Zectran.

Materials and Methods

Application of Zectran. Green sprouting broccoli was grown from seed in a sandy loam–sponge rock (1:1 v./v.) soil mixture in 3-gallon cans with drainage. After 63 days, at the appearance of the first flowers, Zectran insecticide was applied to the plant by dissolving 4 mg. of 4-dimethylamino-3,5-xylyl-α^3-3-C^{14}_2-methylcarbamate in 50 μl. of corn oil and streaking the stem immediately below a flower cluster. After 10 days, the broccoli was harvested.

Counting Procedure. All fractions were counted with an end-window Geiger-Müller tube at infinite thickness for 1024 counts. All of the count rates in this report are in gram-counts per minute. This unit is arrived at by multiplying the net count rate of an infinitely thick sample taken from any given fraction by the total weight in grams of that fraction. This is an arbitrary measure of the total activity in each fraction and served to determine the distribution of radioactivity.

Chromatography. Crude extracts were purified on large-scale paper chromatograms (Whatman No. 3 MM) since, in most cases, the radioactive metabolites were associated with large amounts of interfering material, and initial chromatograms did not usually give well defined bands. Whatman No. 1 paper was used in all other cases. In all identification work, the known compound was run simultaneously with the radioactive metabolite. R_f values and solvent systems used are given in Table I.

Fractionation Procedure. To assess

Table I. R_f Values of Metabolites Found in Broccoli

Metabolites	Solvent System[a] I	II	III	IV	V	VI	VII
4-Dimethylamino-3,5-xylyl methylcarbamate	0.72	0	...	0.78	...	...	...
4-Dimethylamino-3,5-xylenol	0.36	0	...	0.47	0.19	...	...
2,6-Dimethylhydroquinone	...	0.21	...	...	0.53	...	...
2,6-Dimethyl-*p*-benzoquinone	...	0.74	0.97	0.92	0.90	...	...
4-Dimethylamino-3,5-dimethyl-*o*-benzoquinone	0.92	0	0.40	0.97	...	...	...
2,6-Dimethyl-*p*-benzoquinone-2,4-dinitrophenylhydrazone	...	...	...	...	..	0.12	0.19
4-Dimethylamino-3,5-dimethyl-*o*-benzoquinone-2,4-dinitrophenylhydrazone	...	...	...	...	...	0.06	...

[a] I: Isoamyl alcohol–formic acid–water (12:1:7 v./v.). II: Benzene saturated with formic acid. III: Benzene–acetic acid–water (1:2:1 v./v.). IV: Chloroform–acetic acid–water (2:2:1 v./v.). V: Methanol–water (1:1 v./v.) saturated with dibutylphthalate as the mobile phase and dibutylphthalate as the stationary phase. VI: *n*-Heptane saturated with dimethylformamide as the mobile phase and dimethylformamide as the stationary phase. VII: *n*-Heptane saturated with methanol as the mobile phase and methanol as the stationary phase.

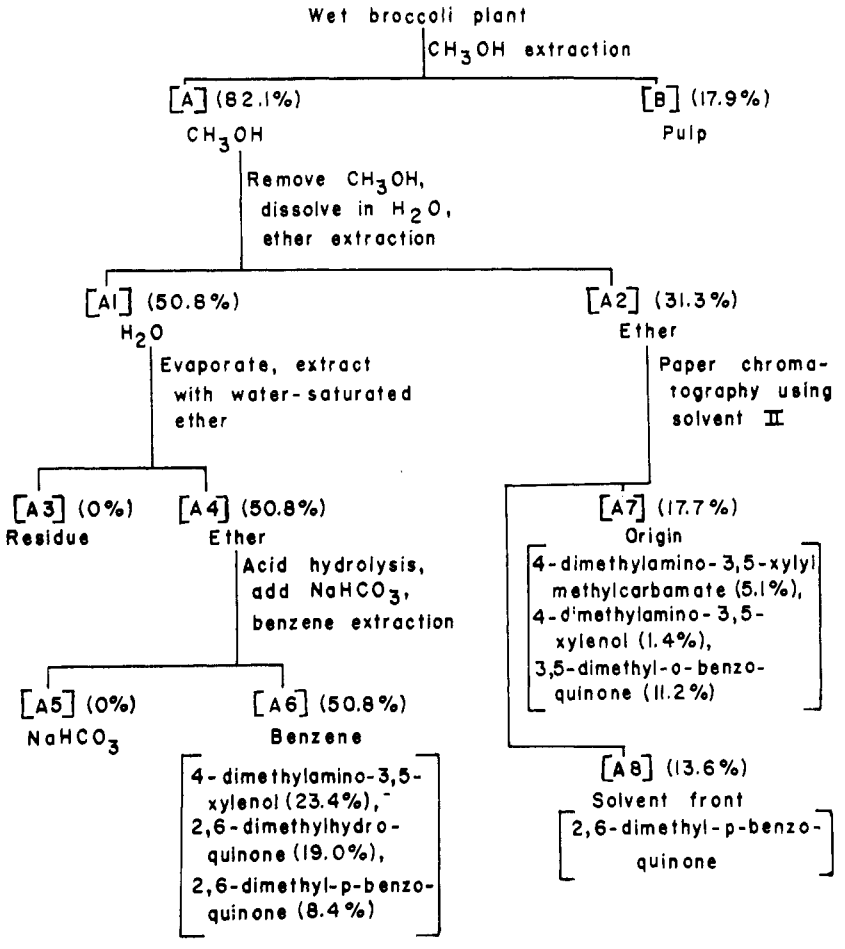

Figure 1. Fractionation procedure used to isolate metabolites from broccoli; methanol-soluble material

The per cent figures indicate the proportion of the total radioactivity initially present in the plant tissue

the suitability of a methanol extraction for removing Zectran, without change, from broccoli tissue, the following experiment was carried out. Broccoli was spiked with radioactive Zectran and immediately extracted for 7.5 hours with methanol. The methanol was removed in vacuo and the residue was chromatographed on a large scale in solvent II. All of the activity was at the origin and could be only Zectran or 4-dimethylamino-3,5-xylenol. A 1-inch strip was cut from the paper and rechromatographed in solvent I. Only Zectran was present. From this it was concluded that methanol extraction did not destroy Zectran.

After harvesting, the treated broccoli was cut into small pieces, immersed in boiling methanol for 15 minutes, and continuously extracted with methanol for 7.5 hours. The fractionation procedure is shown in Figure 1.

The methanol extracts were evaporated in vacuo at <35° C. The residue (*A*), 1.7 grams, contained 544 gram-counts per minute, or 82.1% of the total activity. Pulp (*B*), 2.1 grams, contained 119 gram-counts per minute, or 17.9% of the total activity. Fraction (*A*) was dissolved in water and continuously extracted with peroxide-free ether for 4 hours. (Peroxide-free ether was prepared by washing U.S.P. ether with an aqueous suspension of ferrous hydroxide and then with water.)

Ether-Soluble Material. The ether was dried over anhydrous magnesium sulfate and evaporated in vacuo at <35° C. The residue (*A2*), 0.2 gram, contained 150 gram-counts per minute, or 31.3% of the tota activity. This was separated on a large-scale paper chromatogram with solvent II (Table I).

The activity at the origin (*A7*) represented 17.7% of the total. The activity (*A8*) that moved with the green color to the solvent front represented 13.6%. Methanol elution and paper chromatography of (*A7*) using solvent I established the presence of 4-dimethylamino-3,5-xylyl methylcarbamate as 5.1% of the total activity, of 4-dimethylamino-3,5-xylenol as 1.4%, and of 4-dimethylamino - 3,5 - dimethyl - *o*-benzoquinone as 11.2%. Additional paper chromatography with solvent IV confirmed these findings.

(*A8*) was eluted with benzene and chromatographed on paper using solvent III. The paper was sprayed with 2,4-dinitrophenylhydrazine reagent (saturated solution in 2*N* HCl). The hydrazone was eluted from the paper with ethyl acetate and cochromatographed using solvent VI with the known 2,4-dinitrophenylhydrazone of 2,6-dimethyl-*p*-benzoquinone. This derivative was prepared by spraying filter paper containing the quinone and then eluting with ethyl acetate. The yellow band containing the activity coincided with the known, thereby suggesting that (*A8*) is 2,6-dimethyl-*p*-benzoquinone. Another paper chromatogram of (*A8*) was run using solvent VII. After exposure to ammonia vapor, the blue band, which coincided with the known, contained the activity.

Ether-Insoluble Material. The water that had been extracted continuously with ether was concentrated to dryness in vacuo at <40° C. The residue (*A1*), 0.9 gram, contained 242 gram-counts per minute, or 50.8% of the total activity. This was allowed to equilibrate with atmospheric moisture for 16 hours and then was extracted with water-saturated, peroxide-free ether for 25 hours in a Soxhlet extractor. The ether was dried and evaporated in vacuo. The residue (*A4*) was hydrolyzed at 110° C. with 0.75*N* HC containing 0.25% (v./v.) H_2SO_3 in a sealed tube for 3.5 hours. The reaction mixture was made alkaline with sodium bicarbonate and extracted with benzene.

The hydrolyzed material (*A6*) was chromatographed on paper using solvent II. Of the total activity, 23.4% coincided with 4-dimethylamino-3,5-xylenol, 19.0% with 2,6-dimethylhydroquinone, and 8.4% with 2,6-dimethyl-*p*-benzoquinone. Since the latter does not form conjugates, this material must be an artifact arising from 2,6-dimethylhydroquinone during hydrolysis and work-up. Therefore, 23.4% of the total activity is represented by conjugated 4-dimethylamino-3,5-xylenol, and 27.4% (19.0 + 8.4) by conjugated 2,6-dimethylhydroquinone. Another paper chromatogram of (*A6*) using solvent V confirmed this finding.

Methanol-Insoluble Material. The fractionation procedure for the methanol-insoluble material is shown in Figure 2.

The pulp (*B*) was delignified with acetic acid and sodium chlorite using the procedure of Wise *et al.* (*15*). Centrifugation left 1.2% of the total activity in the precipitate (*B1*). The filtrate was dialyzed in cellophane against running tap water for 25 hours. The lignin fragments (*B2*) remaining in the cellophane contained 1% of the total activity; the remaining 15.7% of the radioactivity was lost during chlorite delignification and in the diffusate during dialysis. The material lost was probably comprised of both low molecular weight lignin fragments and hydrolysis products of hemicellulose.

(*B*2) was hydrolyzed with 0.33*N* H_2SO_4 according to the procedure described by Mo ier-Williams (*11*). Solid barium carbonate was added and the barium sulfate was removed by filtration. The hydrolysis mixture (*B*4) was extracted with ethyl acetate, and (*B*5) was chromatographed on paper using solvents II and III. Two thirds of the activity coincided with 4-dimethylamino-3,5-dimethyl-*o*-benzoquinone, and one third with 2,6-dimethyl-*p*-benzoquinone.

The water phase (*B*6) was dialyzed against water in a beaker, with stirring, for 30 hours. The diffusate was replaced twice with fresh water during the operation. The nondiffusible fraction (*B*8) retained a small amount of activity. Its ultraviolet spectrum in water was almost identical to that published by Herbst (*7*) as shown in Figure 3.

(*B*9), which represents low molecular weight lignin fragments, was submitted to chromatography on ion exchange paper as follows: first, paper with Dowex 50 ion exchange resin (S & S, grade No. 2393); then paper with Dowex 2 (bicarbonate form; S & S, grade No. 2494). The activity (B10) was not held up by Dowex 50 but was by Dowex 2. (*B*11), located at the solvent front, represented nonradioactive colored material that was not held up by the paper with Dowex 2. This material should have contained sugars if (*B*2) had been hemicellulose. However, (*B*11) did not contain detectable sugars as determined by electrophoresis using 2% Borax buffer as described by Robinson and Rathbun (*13*). The ultraviolet spectrum of (*B*10) in water was almost identical to that of (*B*8), again suggesting that it contained lignin fragments.

The pulp (*B*) was hydrogenated according to the procedure of Brown and Neish (*3*). The product was chromatographed on Whatman No. 3 MM paper using solvent II. All of the activity was located at the origin, suggesting the presence of 4-dimethylamino-3,5-dimethyl-*o*-benzoquinone. The activity was removed with hot ethyl acetate. A paper chromatogram using solvent IV had peak activity which coincided with 4 - dimethylamino - 3,5 - dimethyl - *o*-benzoquinone. The 2,4-dinitrophenylhydrazone of this radioactive material was chromatographed on paper using solvent VI. All of the activity corresponded to 4-dimethylamino-3,5-dimethyl - *o* - benzoquinone - 2,4 - dinitrophenylhydrazone.

Synthesis of 4-Dimethylamino-3,5-dimethyl - *o* - benzoquinone. 4-Dimethylamino - 3,5 - dimethylpyrocatechol was prepared using the procedure of Loudon and Scott (*9*). The compound was oxidized to 4-dimethylamino-3,5-dimethyl-*o*-benzoquinone with silver oxide. Bis-2,4-dinitrophenyl-hydrazone was prepared. Anal: calcd. for $C_{22}H_{21}N_9O_8$: N, 23.4; found: N, 23.0.

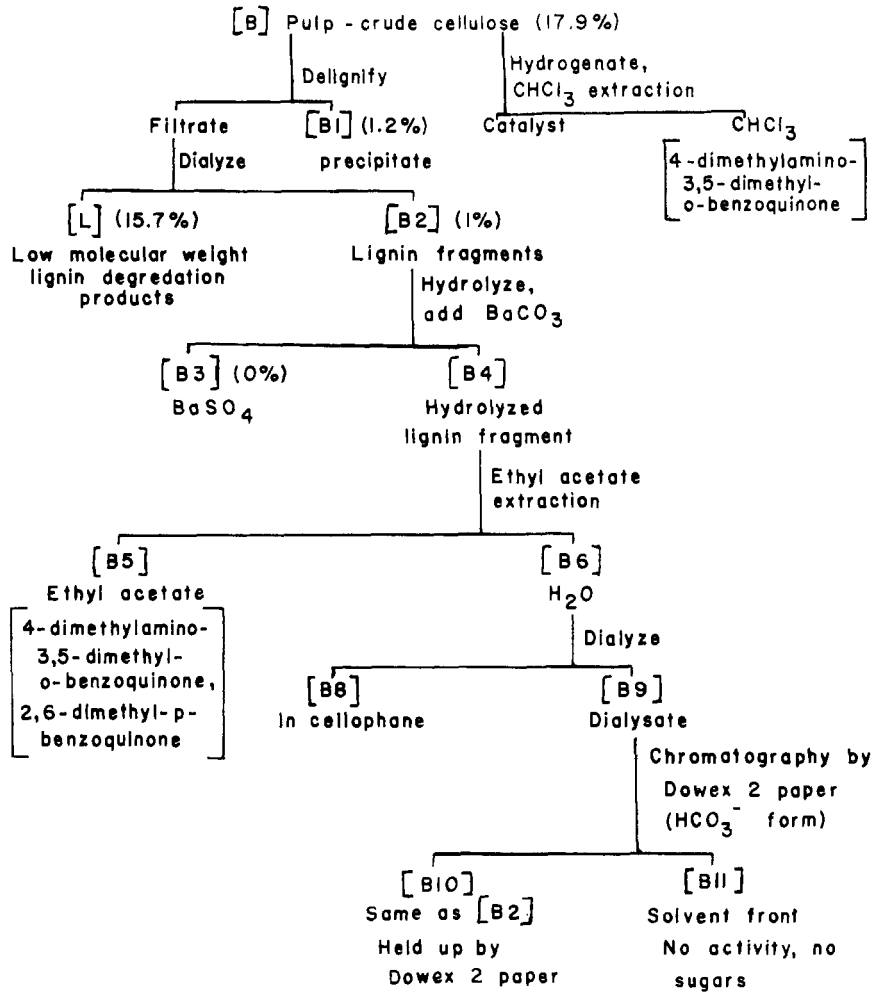

Figure 2. Fractionation procedure used to isolate metabolites from broccoli; methanol-insoluble material

The per cent figures indicate the proportion of the total radioactivity initially present in the plant tissue

Results

Of the total radioactivity, 17.9% is associated with the extracted pulp. The remaining 82.1% is accounted for as shown by the distribution of radioactivity in Table II.

4 - Dimethylamino - 3,5 - dimethylpyrocatechol and 2,6-dimethylhydroquinone, detected as their quinones, have been found to be associated with the lignin.

Discussion

Figure 4 shows the proposed metabolic pathway of Zectran. The sequence of events, as proposed in Figure 4, is not to be construed as unequivocal. The concept that hydrolysis is the initial reaction taking place during the in vivo destruction of carbamate pesticides has been seriously questioned (*4*), but the present work does not resolve this point.

Investigation of the lignin has shown that both 4-dimethylamino-3,5-dimethylpyrocatechol and 2,6-dimethylhydroquinone are present. The compounds were actually detected as their quinones, but there is no evidence in the literature suggesting that quinones are incorporated into lignin. Therefore, the quinones are artifacts arising as a result of the various chemical manipulations performed on the lignin in which the reduced compounds were present. Since lignin, once deposited, does not appear

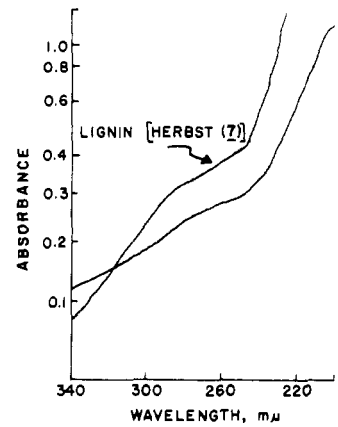

Figure 3. Ultraviolet spectrum of (B8) in water, as compared with published spectrum (*7*)

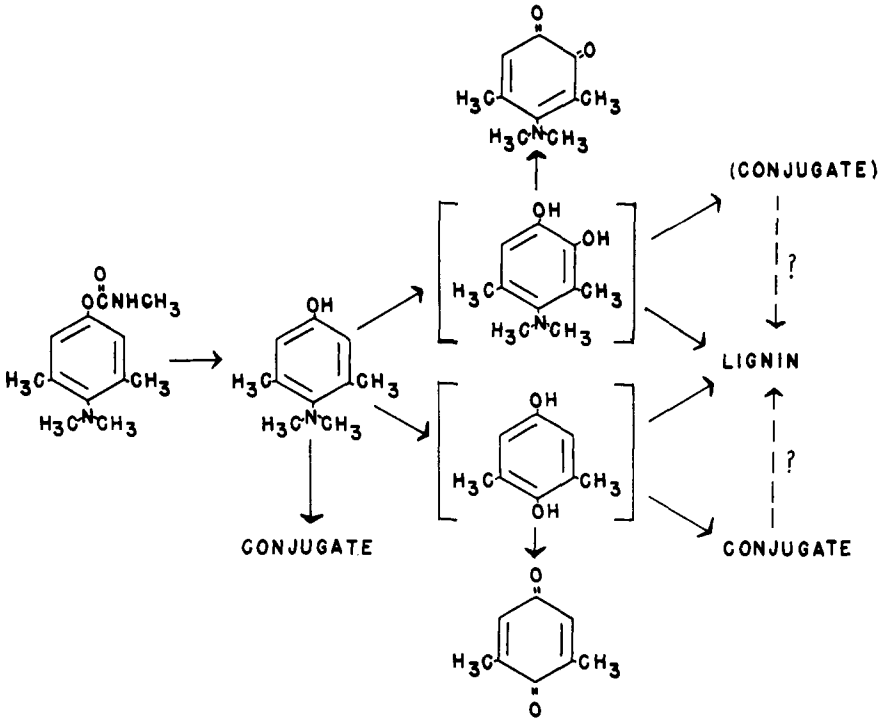

Figure 4. Proposed metabolic pathway of Zectran insecticide in broccoli

to be re-utilized by the plant, it probably serves as a permanent storage facility for reactive toxic groups eventually isolating them from the regions of active growth.

Both 4-dimethylamino-3,5-xylenol and 2,6-dimethylhydroquinone appear as water-soluble conjugates. Together, these two compounds represent 50% of the total radioactive residue. The conjugate of 4-dimethylamino-3,5-dimethylpyrocatechol was not found. The explanation for this lies possibly in the extreme sensitivity of the catechol to oxidation. The catechol could not be isolated from the synthetic reaction product—it seemed to be an equilibrium mixture of oxidized and reduced forms. The spectral changes in the ultraviolet, in response to a change in the oxidative or reductive potential of the solvent, were identical to those arising from 4-*tert*-butylpyrocatechol. The product was oxidized to the quinone and characterized as the 2,4-dinitrophenylhydrazone.

The incorporation of catechol derivatives into lignin has been reported by Brown and Neish (*2*). It is not known if the reactions leading to the transformation of catechol and hydroquinone into lignin residues require that the substrates be in a conjugated form. Accordingly, this uncertainty has been indicated by means of dotted lines in Figure 4. The compounds in brackets in Figure 4 were not detected.

Additional experimentation has shown that quinone was formed from 2,6-dimethylhydroquinone during the mechanical and chemical manipulation involved in this work. Also, from the discussion about the lability of 4-dimethylamino-3,5-dimethylpyrocatechol, none of this compound would be expected to survive these procedures without oxidation and, as expected, none was found. As a result of the foregoing, it is not known how much of these quinones was actually present as a true metabolite and how much was formed during the work-up. That proportion formed during experimentation would be an artifact and would represent the reduced products. The authors felt, from their experiments with the hydroquinone derivative, that not all of the 2,6-dimethyl-*p*-benzoquinone in Table II is an artifact, *i.e.*, some is formed in vivo.

Initially, there was some question as to whether fraction (*B*2) was hemicellulose or lignin fragments. However, in view of the characteristic ultraviolet spectra and the lack of sugars after hydrolysis, this fraction appeared to be a nondiffusible, water-soluble lignin fragment arising during chlorite delignification. Subsequent hydrolysis of (*B*2) degraded it to some extent as shown by the fractionations leading to (*B*5), (*B*8), and (*B*10).

Generally the chlorite delignification process removes small amounts of hemicellulose to the extent of a few per cent on a weight basis (*7*). Since the major portion of hemicellulose still remained with the holocellulose, and the residual radioactivity in the holocellulose was very small, 1.2%, it did not seem likely that the small amount of hemicellulose lost in this procedure could account for any significant portion of the radioactivity lost in fraction (*L*).

The radioactivity remaining in the holocellulose (*B*1) after delignification is thought to be associated with retained lignin. Chlorite delignification does not remove all of the lignin, some 2 to 5% being retained by the holocellulose (*7*). The ratio of the specific activity calculated for lignin to the measured value for holocellulose (*B*1), 26, suggests that 4% of the lignin still remained in (*B*1), as shown in Table III. The cellulose is felt to be inactive.

The total counts lost as a result of chlorite delignification were 181 − 22 = 159. If we add to this the nondiffusible, water-soluble lignin fragments (*B*2), 159 + 10 = 169, we have the total counts due to lignin. This is 93% of the counts present in (*B*) before delignification. In addition, 1.67 − 1.14 = 0.53 gram of lignin was removed. Therefore, the calculated specific activity of the lignin was 169/0.53 = 319 counts per minute per 150 mg. sample. The calculated specific activity of the lignin is larger than that of the holocellulose (*B*1) by a factor of 319/12.3 = 26. This suggests that 4% of the radioactive lignin still remains in the holocellulose.

Table II. Distribution of Radioactivity in Broccoli Treated with 4-Dimethylamino-3,5-xylyl Methylcarbamate

Metabolites	*Total Activity, %*
4-Dimethylamino-3,5-xylyl methylcarbamate	5.1
4-Dimethylamino-3,5-xylenol	1.4
4-Dimethylamino-3,5-dimethyl-*o*-benzoquinone	11.2
2,6-Dimethyl-*p*-benzoquinone	13.6
Conjugated 4-dimethylamino-3,5-xylenol	23.4
Conjugated 2,6-dimethylhydroquinone	27.4
Lignin	17.9

Table III. Radioactivity Present in Crude Cellulose before and after Chlorite Delignification

Material	*Weight, Grams*	*Specific Activity, C.P.M. per 150 Mg. of Sample*	*Total Activity, Gram-Counts per Minute*
(*B*)	1.67	108.1	181.0
(*B*1)	1.14	10.8	12.3
(*B*2)	0.37	27.0	10.0

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ANIMAL METABOLISM OF INSECTICIDES

Identification of Metabolites of Zectran Insecticide in Dog Urine

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The metabolic study of Zectran in dog urine is described. Free 4-dimethylamino-3,5-xylenol and water-soluble conjugates of 4-dimethylamino-3,5-xylenol and 2,6-dimethylhydroquinone are identified as metabolites.

THE EFFECTIVENESS of Zectran (Registered trade-mark of The Dow Chemical Co.), 4-dimethylamino-3,5-xylyl methylcarbamate, for the control of mollusks and arthapod pests of plants is well documented (*7*). The potential widespread use of this compound raises a problem concerning the manner in which an animal can detoxify and eliminate the ingested pesticide from the body. The present investigation aims at identifying the metabolites of Zectran in dog urine.

Materials and Methods

Feeding. A 5-month old male beagle was preconditioned by feeding 6 mg. (20 p.p.m.) of inactive Zectran twice daily in his rations, which consisted of Purina Dog Chow (Ralston-Purina Co., St. Louis, Mo.). The inactive Zectran was replaced by 4-dimethylamino-3,5-xylyl-α^3, 3-$C^{14}{}_2$ methylcarbamate, specific activity 0.031 mc. per mmole. The dog was fed 150 grams of rations containing 20 p.p.m. labeled Zectran twice daily for 7 days. At the end of this time, the labeled Zectran was replaced by inactive material and feeding was continued.

Chromatography and Counting Procedures. These procedures are the same as those already described by Williams *et. al.* (*7*). R_f values and solvent systems are given in Table I.

Fractionation. Urine collections were commenced at the time the feeding of the radioactive material started and continued for 5 days after the feeding ceased, at which time no more radioactivity was present. The samples were stored in covered jars at 4° C. The urine samples were combined and concentrated in vacuo at <40° C. to 400 ml. The concentrated urine was adjusted to pH 6.6 with 1*N* HCl (Figure 1) and continuously extracted with peroxide-free ether (34° C.) for 16 hours. (Peroxide-free ether was prepared by washing U.S.P. ether with an aqueous suspension of ferrous hydroxide and then with water.) The ether extract [1] contained 8.4% of the total activity. The aqueous phase [2] contained 91.6%.

Ether Extract [1]. The fractionation procedure for the ether-soluble material is shown in Figure 1.

The ether [1] was dried over anhydrous magnesium sulfate and concentrated in vacuo at <40° C. to 50 ml. The ether was extracted four times with equal volumes of 10% sodium bicarbonate. The ether was dried and concentrated to a sirup [1*A*] as above. The residue contained 4.7% of the total activity.

The sodium bicarbonate extracts were adjusted to pH 6.6 with 1*N* HCl and continuously extracted with peroxide-free ether for 16 hours. The ether was dried and concentrated in vacuo. The residue [1*B*] contained 3.7% of the total activity.

Separation of Fraction [1*A*]. Fraction [1*A*] was submitted to large-scale

Table I. R_f Values of Metabolites Found in Dog Urine

Metabolites	Solvent System[a] I	II	III	IV	V	VI	VII	VIII	IX	X	XI
4-Dimethylamino-3,5-xylyl methylcarbamate	0.64	0	0.06	0.93	0.96	0.93	0.96	0.95	0.94	0.49	0.22
4-Dimethylamino-3,5-xylenol	0.37	0	0.02	0.86	0.93	0.84	0.96	0.96	0.96	0.28	0.18
2,6-Dimethylhydroquinone	0.91	0.24	0.91	0.83	0.80	...	0.75	0.58	0.91	0.56	...
2,6-Dimethyl-*p*-benzoquinone	0.96	0.90	0.82	1.00	0.94	...	0.96	0.93	0.96	...	0.64
4-Dimethylamino-3,5-xylyl sulfate	0.15	0.34	0.86	...	0.80	...	0.07	0	0.51	0.26	0.64

[a] I: Isoamyl alcohol–formic acid–water (12:1:7 v./v.). II: benzene saturated with formic acid. III: benzene–acetic acid–water (1:2:1 v./v.). IV: chloroform–acetic acid–water (2:1:1 v./v.). V: *n*-butanol saturated with 1.5*N* ammonia. VI: ethanol–ammonia–water (10:1:4 v./v.). VII: *n*-butanol–benzene–water (1:9:10 v./v.). VIII: *n*-butanol–benzene–water (1:19:20 v./v.). IX: *n*-butanol–ethanol–water (17:3:20 v./v.). X: cyclohexane saturated with dimethylformamide as the mobile phase and dimethylformamide as the stationary phase. XI: methanol–water (1:1 v./v.) saturated with dibutylphthalate as the mobile phase and dibutylphthalate as the stationary phase.