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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.

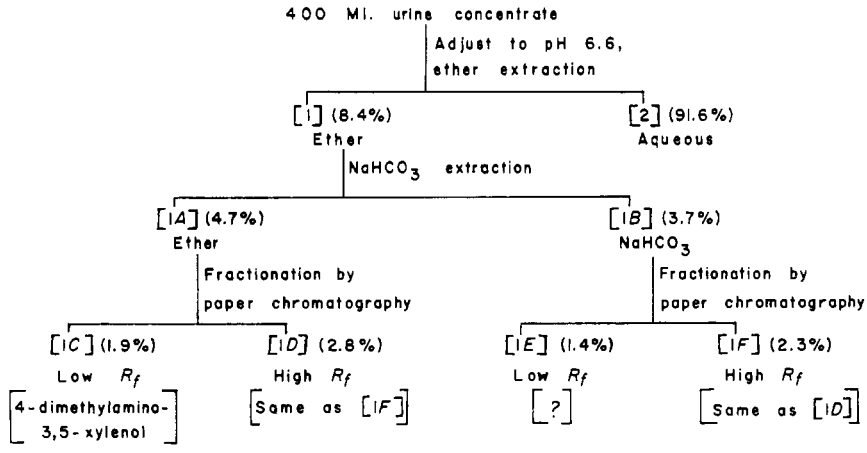

Figure 1. Fractionation procedure used to isolate metabolites from dog urine: ether-soluble material

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

paper chromatography using solvent I (Table I). R_f 0 to 0.23 contained no activity. R_f 0.23 to 0.48 contained 1.9% of the total activity. This was eluted from the paper with methanol and concentrated to dryness in vacuo at <40° C. to give residue [1C]. R_f 0.48 to 1.00 contained 2.8% of the total activity. This was treated as above to give fraction [1D].

Identification of Fraction [1C]. Paper chromatograms of [1C] were run with solvents III and X. On both papers the activity coincided with 4-dimethylamino-3,5-xylenol.

Identification of Fraction [1D]. On a large-scale paper chromatogram of [1D] using solvent V, the activity was located at R_f 0.79 to 1.00. This was eluted with methanol, concentrated, and rerun as above with solvent IV. The activity, located at R_f 0.69 to 1.00, was eluted with methanol and concentrated. Seven paper chromatograms were run as shown in Table II.

Fraction [1D] has not been identified. Subsequent work will show that it is the same material as one of the components of fraction [1B].

Identification of Fraction [1B]. On a large-scale paper chromatogram of [1B] using solvent I, the activity was located at R_f 0.65 to 1.00. This was eluted with methanol and concentrated. The apparent electrophoretic mobility of the material was zero. (Electrolyte, 0.75*M* formic acid; ambient temperature; 13 volts per cm.; time, 2 hours.) The material was rechromatographed on a large scale with solvent V. The activity, located at R_f 0.72 to 0.96, was eluted with methanol and concentrated. Paper chromatograms were run using solvents I, II, VII, VIII, and XI. On each paper there were two radioactive entities. On all papers, fraction [1E], 1.4% of the total activity, had lower R_f values than fraction [1F], 2.3%. Comparison of the R_f values of fractions [1D] and [1F] suggests that these two entities are identical as shown in Table II.

The remainder of fraction [1B] was hydrolyzed in a sealed tube with 1.5*N* HCl, 0.01*M* in $SnCl_2$. The hydrolysis mixture was made alkaline with sodium bicarbonate and extracted with peroxide-free ether. A paper chromatogram run with solvent II had activity at R_f 0.12 to 0.17 and R_f 0.26 to 0.31. These correspond to the R_f values of [1E] and [1F]. Therefore, hydrolysis did not occur. The two radioactive components, (1D and 1F) and (1E), remain unidentified.

Hydrolysis of Water-Soluble Portion. Part of the water-soluble residue [2] was hydrolyzed with 6*N* HCl, 0.05*M* in $SnCl_2$, at 100° C. for 3 hours in a sealed tube. The hydrolysis mixture was made alkaline with sodium bicarbonate and extracted with peroxide-free ether.

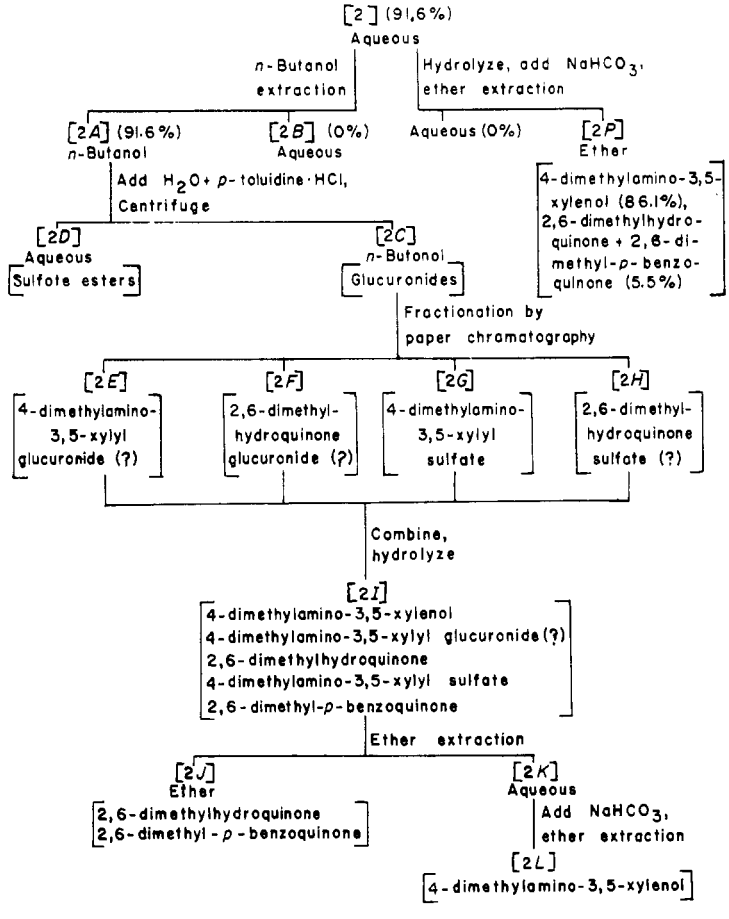

Figure 2. Fractionation procedure used to isolate metabolites from dog urine: *n*-butanol-soluble material

The per cent figures indicate the proportion of radioactivity initially present in the urine

Table II. R_f Values of Unidentified Ether Soluble Metabolites in Dog Urine

Solvent System	Fraction [1E]	[1F]	[1D]
I	0.16	0.84	0.84
II	0.15	0.29	0.28
III	...	...	0.54
V	0.16	0.82	...
VII	0.47	0.86	0.85
VIII	0.16	0.75	0.80
X	...	...	0.50
XI	0.07	0.66	0.64

Paper chromatograms of the ether-soluble material [2P] (Figure 2) were run using solvents II and III. Of the activity present, 94% in fraction [2P] coincided with 4-dimethylamino-3,5-xylenol, 2% with 2,6-dimethylhydroquinone, and 4% with 2,6-dimethyl-*p*-benzoquinone. As 2,6-dimethyl-*p*-benzoquinone does not form conjugates, it is an artifact which arises as a result of oxidation of 2,6-dimethylhydroquinone. Therefore, the only metabolites found after hydrolysis were 4-dimethylamino-3,5-xylenol (86.1% of the total activity) and 2,6-dimethylhydroquinone (5.5%). These were present in the urine as water-soluble conjugates.

Electrophoresis of Water-Soluble Material. Part of the water-soluble material [2] was submitted to large-scale paper chromatography using solvent I. There were three areas of activity; fraction 1 (R_f 0 to 0.23), fraction 2 (R_f 0.23 to 0.40), and fraction 3 (R_f 0.40 to 1.00). Each fraction was submitted to paper electrophoresis as follows: electrolyte, 0.025*M* potassium acid phthalate buffer adjusted to pH 5.9 with 0.2*N* NaOH; ambient temperature; 13 volts per cm.; time, 6 hours. Figure 3 shows the apparent electrophoretic mobility of the radioactivity in these fractions.

The radioactive areas in fraction 2 are tentatively identified as follows: The area from +15 to +12 cm. represents strongly acidic sulfate esters; +5 to +2 cm. should be mildly acidic material, such as 2,6-dimethylhydroquinone glucuronide; +1 to −1 cm. is the area in which neutral compounds such as 4-dimethylamino-3,5-xylyl glucuronide would remain; and finally, the area from −3 to −5 cm. represents compounds which have some basic character as might be represented by the glucuronolactone of 4-dimethylamino-3,5-xylenol. Glucuronolactones frequently are formed from glucuronides in the process of evaporation to dryness. Fraction 1 contained most of the glucuronide derivatives, and fraction 3 contained only sulfate esters.

Water-Soluble Material. The fractionation procedure for [2] is shown in Figure 2.

The water-soluble portion [2] contained 91.6% of the total activity. This was adjusted to pH 8.5 with 20% NaOH (w. v.) and extracted seven times with 80-ml. portions of *n*-butanol by shaking the mixtures for 20 minutes on a mechanical shaker, centrifuging, and drawing off the solvent. The *n*-butanol extracts were combined and concentrated in vacuo at <50° C. The aqueous solution [2B] contained no activity.

Isolation of Glucuronides (*6*). The residue [2A] remaining as a result of the concentration of the *n*-butanol solution was dissolved in 100 ml. of water and 4 grams of *p*-toluidine hydrochloride were slowly added with stirring at 60° to 70° C. The solution was allowed to stand at 4° C. for 16 hours. The mixture was centrifuged and the oily bottom layer (2C) was taken up in 50 ml. of water. One-half gram of decolorizing carbon was added and the mixture was heated and filtered hot. The filtrate was cooled but no crystals formed. The solution was concentrated, the glucuronide was regenerated with 5 ml. of concentrated ammonia, and the solution was evaporated to dryness.

A large-scale paper chromatogram using solvent I was run on the residue [2C]. The activity was spread over the length of the paper. The chromatogram was cut at R_f 0.10, 0.26, and 0.61, and each of the four fractions (2E, 2F, 2G, and 2H) was eluted from the paper with methanol and concentrated. Paper chromatography of each fraction with solvent IX indicated the presence of four separate compounds; [2E] contained activity located at R_f 0.11 to 0.15, [2F] at R_f 0.09 to 0.13 and 0.45 to 0.49, [2G] at R_f 0.47 to 0.51 and 0.67 to 0.71, and [2H] at R_f 0.68 to 0.72 and 0.83 to 0.87. These data show that there was overlapping in adjacent fractions.

Fractions (2E, 2F, 2G, and 2H) were combined and hydrolyzed at 100° C. for 3 hours with 1.5*N* HCl, 0.01*M* in $SnCl_2$. Paper chromatography of [2I] with solvent II gave the following distribution of activity: R_f 0 to 0.02, (4-dimethylamino-3,5-xylenol), R_f 0.06 to 0.11 (probably represents unhydrolyzed glucuronides), R_f 0.20 to 0.25 (2,6-dimethylhydroquinone), R_f 0.29 to 0.34 (unhydrolyzed 4-dimethylamino-3,5-xylyl sulfate), and R_f 0.85 to 0.89 (2,6-dimethyl-*p*-benzoquinone).

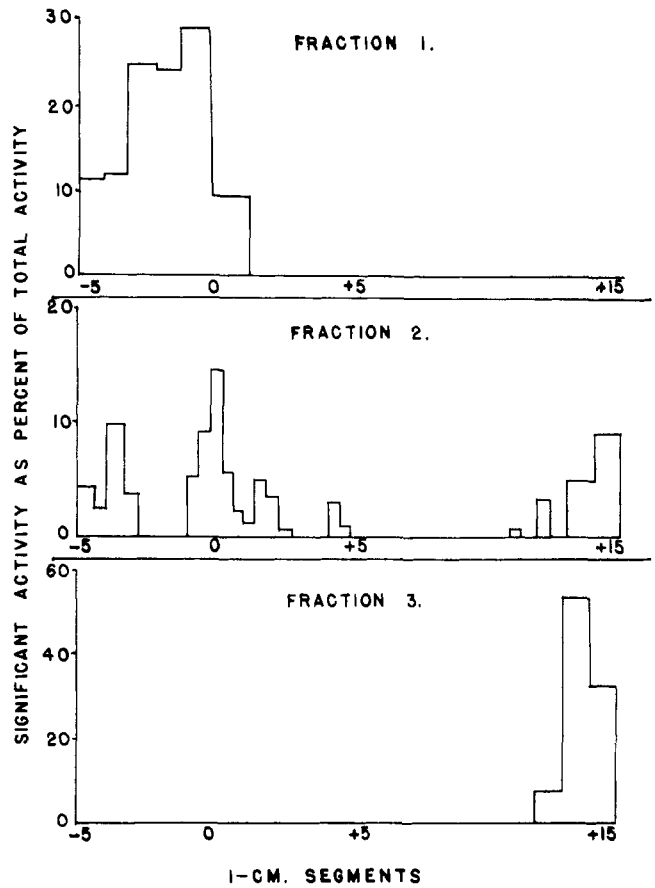

Figure 3. Paper electrophoresis of [2] in 0.025M phthalate buffer at pH 5.9, 6 hours

The hydrolyzed material was extracted with peroxide-free ether and [2J] was chromatographed on paper with solvent II. The activity coincided with 2,6-dimethylhydroquinone and 2,6-dimethyl-*p*-benzoquinone. This quinone is an artifact as explained by Williams *et al.* (*7*). The acid solution [2K] was made alkaline with sodium bicarbonate and extracted with peroxide-free ether. Paper chromatograms of [2L] were run using solvents I, X, and XI. On each paper the activity coincided with 4-dimethylamino-3,5-xylenol.

Identification of Sulfates. The fractionation procedure for the water-soluble material [2D] is shown in Figure 4.

The top phase [2D] resulting from the centrifugation during the glucuronide isolation was made alkaline with ammonia and extracted three times with chloroform to remove the *p*-toluidine. The aqueous phase was concentrated in vacuo at <50° C. to give a dark, oily residue [2M]. A large-scale paper

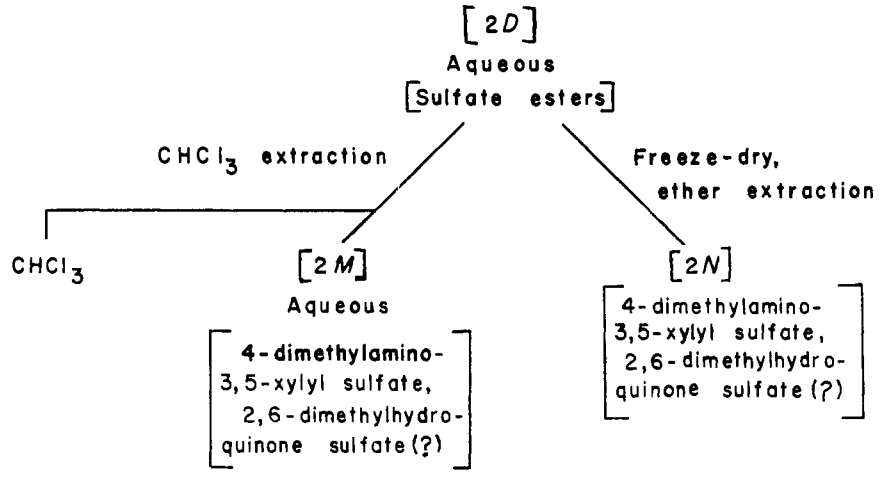

Figure 4. Fractionation procedure used to identify sulfate esters in dog urine

chromatogram of [2*M*] was run with solvent I. The activity, located at R_f 0.20, was eluted with water. Paper chromatograms run with solvents I and IX each contained two areas of activity. One area coincided with 4-dimethylamino-3,5-xylyl sulfate. The second radioactive entity, with higher R_f values, was thought to be 2,6-dimethylhydroquinone sulfate because when hydrolyzed, [2*D*] yielded the xylenol and hydroquinone derivatives.

Ether Extraction of Sulfates. Part of the water-soluble material [2*D*] was concentrated to incipient dryness, freeze-dried, allowed to equilibrate with the air at room temperature, and continuously extracted with peroxide-free, water-saturated ether for 16 hours. Paper chromatograms of [2*N*] using solvents VI and IX each contained two radioactive areas. One coincided with 4-dimethylamino-3,5-xylyl sulfate, and the other area, with higher R_f, is postulated to be 2,6-dimethylhydroquinone sulfate.

Synthesis of 4-Dimethylamino-3,5-xylyl Sulfate. This compound was synthesized according to the procedure of Bernstein and McGilvery (*1*), m.p. 202–203° C. *Anal*: calcd. for $C_{10}H_{15}NO_4S$: N, 5.71, found: N, 5.75.

p-Nitrobenzenediazoniumfluoroborate in acetone, as described by Freeman (*4*), was used to detect 4-dimethylamino-3,5-xylyl sulfate. The procedure used here was a simplification in that the paper was sprayed, dried, and held over ammonia to develop the spot.

Results

Fractionation of the dog urine led to the distribution of radioactivity shown in Table III. The Zectran insecticide was hydrolyzed to 4-dimethylamino-3,5-xylenol; 86.1% was conjugated, 1.9% was excreted as the free xylenol, and 5.5% underwent demethylation and was converted to 2,6-dimethylhydroquinone, which was excreted as a water-soluble conjugate. To the authors' knowledge, this is the first reported example of the conversion of a *p*-aminophenol to a hydroquinone derivative by the dog. The remaining 6.5% was excreted as unidentified, unconjugated ether-soluble material.

Table III. Distribution of Metabolites of Zectran Insecticide in Dog Urine

Metabolites	*Total Activity, %*
Conjugated 4-dimethylamino-3,5-xylenol	86.1
Conjugated 2,6-dimethylhydroquinone	5.5
Unidentified, unconjugated nonacidic material	5.1
4-Dimethylamino-3,5-xylenol	1.9
Unidentified, unconjugated acidic material	1.4

Discussion

The counting of urine and fecal material at infinite thickness showed that approximately one fourth of the excreted radioactivity appeared in the feces and three fourths in the urine. In view of this information, together with past experience in the futility of fecal analysis, attention was turned exclusively to the urinary products. Figure 5 shows the proposed metabolic pathway of Zectran.

The expected reaction of carbamates in vivo in animals is hydrolysis of the ester group to carbamic acid and an alcohol. The former can then be degraded further and the latter oxidized and/or conjugated. However, Casida *et al.* (*2*) feel that hydrolysis by esterases of animal origin does not appear to adequately account for the biological instability of the majority of the carbamate cholinesterase inhibitors. Furthermore, recently (*3*) a demethylation reaction, involving first the formation of an N-methylol derivative followed by loss of the methyl group as formaldehyde, has been suggested as the major initial reaction rather than hydrolysis. However, Hodgson and Casida (*5*) report that Zectran and its hydrolysis product, 4-dimethylamino-3,5-xylenol, each yields the same amount of formaldehyde when acted on by rat liver microsomes. This suggests that demethylation is occurring with Zectran but only at the 4-dimethylaminophenyl group, and would seem to rule out any significant contribution due to oxidation at the *N*-methyl group on the other end

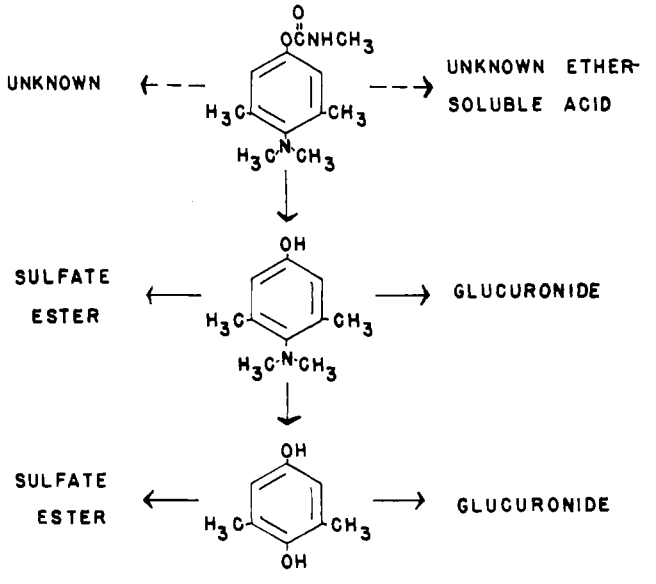

Figure 5. Proposed metabolic pathway of Zectran in the dog

of the mylecule. Indeed, as pointed out, demethylation of the aromatic amine has occurred as shown by the detection of the corresponding hydroquinone derivative.

No unchanged 4-dimethylamino-3,5-xylyl methylcarbamate was found in the urine. The only unconjugated metabolite identified was 4-dimethylamino-3,5-xylenol.

Other ether-soluble radioactive substances were detected but they remain unidentified. Fractions [1*D*] and [1*F*] of Figure 1 could be identical, and chromatographic evidence (Table II) strongly suggests this to be the case. The compound represented by these two fractions is not a carboxylic acid or conjugated sulfate ester since it had an apparent distribution coefficient between ether and 10% sodium bicarbonate solution of 5.3. Any acidic material would have had a coefficient of zero. Fraction [1*E*], an acidic material, might possibly be 6-dimethylamino-3,5-cresotic acid.

In the animal body, phenols undergo two main reactions: conjugation of the hydroxyl group to form both glucuronides and ethereal sulfates. Since hydrolysis of the water-soluble radioactive material resulted in the identification of 4-dimethylamino-3,5-xylenol and 2,6-dimethylhydroquinone, both of these phenols may be present as these conjugated forms. Electrophoretic mobilities of the water-soluble compounds are consistent with the above interpretation.

There is no evidence for the presence of 4 - dimethylamino - 3,5 - dimethylpyrocatechol, a metabolite found in broccoli as a result of Zectran treatment (*7*), in the urine. Apparently, there is no tendency for the dog to hydroxylate this highly substituted aromatic ring to any significant extent. If this had occurred, it should have been possible to find it as a conjugate in the urine.

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INSECTICIDE RESIDUES

The Colorimetric Determination of o-Isopropoxyphenyl-*N*-methylcarbamate

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A method for the determination of o-isopropoxyphenyl-*N*-methylcarbamate residues on various surfaces immediately after spraying and at intervals thereafter has been developed. The insecticide was diazotized with 3-nitroaniline-4-sulfonic acid and determined spectrophotometrically at 490 mμ. The color that developed was very stable in water and obeyed the Beer-Lambert law over the range tested. The method was adapted for the estimation of Sevin, Isolan, Pyrolan, Dimetilan, and Hercules AC-5727.

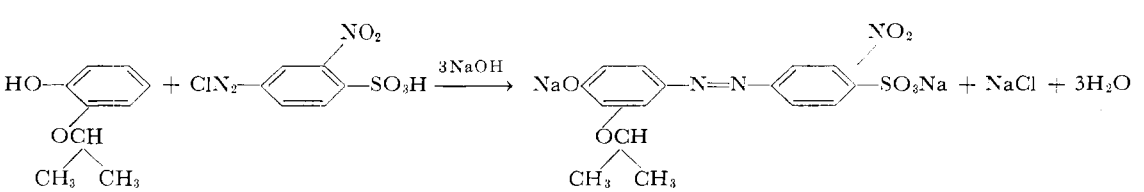
Since the introduction of the first commercial carbamic acid ester, Sevin, considerable interest has been aroused in the use of compounds belonging to this group for pest control. Bayer 39007 (*o*-isopropoxyphenyl-*N*-methylcarbamate) has shown a high degree of activity against mosquitoes and flies. During trials with this compound as a possible insecticide in an antimalarial campaign, it became necessary to estimate residues of the substance on various surfaces. No method was recorded in the literature for the determination of this compound, but other carbamate insecticides were usually determined by the estimation of the phenol obtained upon hydrolysis, by coupling it with *p*-nitrobenzenediazonium fluoroborate (*5*), or 4-amino antipyrine (*2*); by the determination of the aliphatic or aromatic amine found upon hydrolysis (*6*); and by cholinesterase-inhibition methods (*1*).

A simplified procedure has been found in this laboratory for the estimation of microgram quantities of *o*-isopropoxyphenyl-*N*-methylcarbamate, and its possible application to the determination of other compounds is now suggested.

o - Isopropoxyphenyl - *N* - methylcarbamate was dissolved in methanol and hydrolyzed with dilute aqueous sodium hydroxide solution. The *o*-isopropoxyphenol obtained was coupled with 3-nitroaniline-4-sulfonic acid (*4*). In alkaline solution, a red dye (I) was obtained in accordance with the equation.

(I) was stable in water and very soluble, due to its double sodium salt character. Thus, a great advantage was achieved over the colorimetric methods which used organic solvents in the color development stage. Results showed good agreement with the Beer-Lambert law, and were accurate and reproducible.

Five other carbamic acid esters were tested—Sevin, Dimetilan (dimethyl 3-



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