Degradation of ^{14}C-labeled Diazinon in the Rat

W. Mücke,[1] K. O. Alt, and H. O. Esser[2]

The metabolism of Diazinon ^{14}C-labeled in the pyrimidine ring and in the ethoxy groups was investigated in rats. The rapid excretion of the insecticide and its metabolites in both males and females was demonstrated by the time interval of approximately 12 hours for the excretion of 50% of the radioactivity applied. Of this, 69 to 80% were excreted in the urine, 18 to 25% in the feces. No cleavage of the pyrimidine ring with subsequent oxidation of the fragments to CO_2 took place. Any accumulation of radioactivity in the essential organs after feeding Diazinon for 10 days was excluded. Among the four metabolite fractions of urine and feces, three metabolites representing approximately 70% of the radioactivity totally applied were identified in addition to trace amounts of unchanged Diazinon in the feces. Hydrolysis of the ester bond yielding 2-isopropyl-4-methyl-6-hydroxypyrimidine and oxidation at the primary and tertiary C-atom of the isopropyl side chain were found as the main degradative mechanisms. The two main metabolites no longer inhibit cholinesterase and their acute oral toxicities are less than one tenth of that of the parent compound. The position of the three main metabolites on the general pathway was demonstrated by following their metabolism after intravenous application.

Investigations on the metabolic fate of Diazinon [*O,O*-diethyl-*O*-(2-isopropyl-4-methyl-6-pyrimidinyl)-phosphorthioate] in mammals were initiated mainly by the widespread use of this phosphorus ester as an ectoparasiticide in ruminants. These include a series of residue studies in fat and milk of cows (Bourne and Arthur, 1967; Claborn *et al.*, 1963; Derbyshire and Murphy, 1962; Matthysse and Lish, 1968) and sheep (Harrison and Hastil, 1965; Matthysse *et al.*, 1968) after the insecticide had been applied regularly by spraying and dipping or by feeding the animals on pasture treated with the insecticide. Only trace amounts, if at all, of very short-lived Diazinon residues have been found in fat and milk whereas the other tissues were free of the insecticide. In two early studies in the cow (Robbins *et al.*, 1957) and in the goat (Vigne *et al.*, 1957), the rapid and complete elimination of the ^{32}P-labeled insecticide in urine, feces, blood, and milk was shown demonstrating the urine as the main route of excretion.

The distribution and the excretion of ^{32}P-labeled insecticide was also followed in the dog (Millar, 1963) and in the guinea pig (Kaplanis *et al.*, 1962). Here again the considerable breakdown of Diazinon in these species was confirmed. In the case of the guinea pig, the elimination of the radioactivity was terminated within 7 days, whereby more than 87% of the dose orally applied left the body with the urine.

The question about the basis of its relatively low mammalian toxicity caused a series of studies where the type and the rate of detoxication of Diazinon in mammals and insects have been compared. The hydrolysis of the ethoxy phosphorus bond of Diazinon in the rat was found to occur to a much smaller extent as with methoxy type phosphorus esters, whereas in the cockroach very little hydrolysis of the alkyl phosphate bond was observed (Plapp and Casida, 1958). In the same study, amounts of diethylthiophosphate up to 71% were determined, indicating a high rate of hydrolysis of the pyrimidinyl phosphate bond without prior oxidation of the thiono sulfur. On the other hand, such an oxidation, reflected by the simultaneous presence of diethyl phosphate in these experiments, was confirmed in *in vitro* experiments with rat liver microsomes using cholinesterase inhibition tests (Dahm *et al.*, 1962).

The relationships between the oxidation of the thiono sulfur, *i.e.*, a toxication yielding Diazoxon, and the hydrolysis especially at the pyrimidinyl phosphorus bond, *i.e.*, a detoxication, were comparatively investigated in insects and mice (Krueger *et al.*, 1960). Diazinon selectivity was attributed to high levels of Diazoxon in the susceptible species since mice showed a much greater capacity to hydrolyze Diazinon and Diazoxon than the cockroach.

The enzymatic basis of these mechanisms was studied in detail with *in vitro* systems of rat liver and fat body of cockroaches (Fukunaga, 1967; Fukunaga *et al.*, 1967). Three types of enzymatic attack were observed: In the cytoplasmic fraction of both species a glutathione dependent enzyme hydrolyzing Diazinon directly to diethylthiophosphate and "pyrimidinol" derivative; in the microsomal fraction of both species an enzyme oxidizing Diazinon to Diazoxon with the aid of an O_2/$NADPH_2$ system; finally in the rat liver microsomal fraction a Ca^{2+} activated esterase hydrolyzing efficiently Diazoxon.

In addition, the observations of Plapp and Casida concerning the hydrolysis of the ethoxy phosphorus bond were confirmed. An enzyme system of rat liver cytoplasm cleaved the methyl phosphate bond of methyl parathion, methyl paraoxon, and sumithion by the aid of reduced glutathion whereas des-ethyl parathion was produced only very slightly (Fukunaga, 1967).

Summarizing the results obtained so far, it became obvious that, as in the case of the phosphorus moiety, an intimate knowledge of the fate of the pyrimidine heterocycle in mammals should be at one's disposal, too. For this purpose, pyrimidine ring and side chain ^{14}C-labeled Diazinon were prepared and the fate of these differently labeled molecules was followed in the rat. The work presented in this paper summarizes the results obtained from studies on the excretion balance, the distribution in different organs, and on the elucidation of structures and properties of the main metabolites.

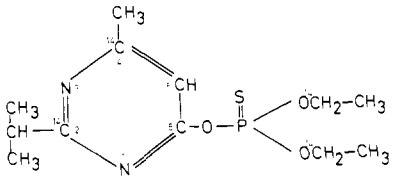
Agricultural Chemicals Research Department, J. R. Geigy S. A., Basle, Switzerland

[1] Present address, Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Mass. 02139

[2] To whom requests for reprints should be sent.

MATERIAL AND METHODS

Labeled Materials. The different types of ^{14}C-labeled Diazinon were synthesized by D. Ryskiewich, Analytical Research Department, Geigy Chemical Corp. Ardsley, N. Y.



(1) 2-^{14}C-Diazinon, specific radioactivity: 4.0 μCi./mg.
(2) 4-^{14}C-Diazinon, specific radioactivity: 2.6 μCi./mg.
(3) Ethoxy-^{14}C-Diazinon, spec. radioactivity: 3.2 μCi./mg.

Animal Experiments. Wistar WU rats of approximately 200-g. body weight were used in all experiments. The animals were kept in all-glass metabolism cages. Feed and water were offered *ad libitum*. The materials were applied in form of water–ethanol solutions (8:2 V./V.) orally by stomach tube or intravenously into the tail vein.

The urine used for the isolation of metabolites was received from male rats which were fed daily with 1 mg. of Diazinon on four subsequent days (ratio of 2-^{14}C-labeled and unlabeled material 1:10). The radioactivity of the urine was measured directly in scintillation cocktail 1.

The CO_2 of the expired air was absorbed into 3*M* sodium hydroxide solution, recaptured into ethanolamine–methanol solution (6:44 V./V.) after acidification with sulfuric acid and counted in scintillation cocktail 2.

The feces were homogenized and extracted 3 times with methanol–acetone (1:1 V./V.). The extract was evaporated to dryness in vacuum, redissolved in methanol–toluene (1:1 V./V.) and assayed in scintillation cocktail 1.

The residue was dried and its radioactive content was determined in scintillation cocktail 2 after combustion according to Kalberer and Rutschmann (1961).

The pattern of metabolites in urine and extracts of feces were determined by TLC in system 1. The radioactive zones of the plates were scrapped off and counted in scintillation cocktail 3.

In the organ distribution study, the animals were sacrificed at definite time intervals after the end of the application period. The organs were immediately dissected. Samples of fat were collected from the intestinal, subcutaneous, and testis fat deposits. Samples of muscles were drawn from back, foreleg, and hindleg. All samples, except fat, were refrigerated immediately and homogenized with 4 volumes of water. The homogenates were lyophilized and aliquots of the residues were combusted according to Kalberer and Rutschmann. The fat samples were treated in the same manner as described for feces. Muscles and fat were calculated as 39% and 14% of the body weight, respectively.

Separation and Isolation of the Metabolites. Thin layer chromatography (TLC) was performed on Silica gel G (Merck) 0.25 mm. The following solvent systems were used:

1. Ethyl acetate–ethanol–ammonia 80:15:5
2. Toluene–acetic acid–H_2O 60:60:6
3. Benzene–ethanol 9:1
4. Dioxane–ethanol–H_2O 5:3:1
5. Ethanol–ethylacetate–acetic acid 3:4:2
6. Acetonitrile–ammonia–H_2O 40:9:1
7. Benzene–chloroform–ethylacetate–propanol 4:2:4:2

Counter current distribution (CCD) used for the purification

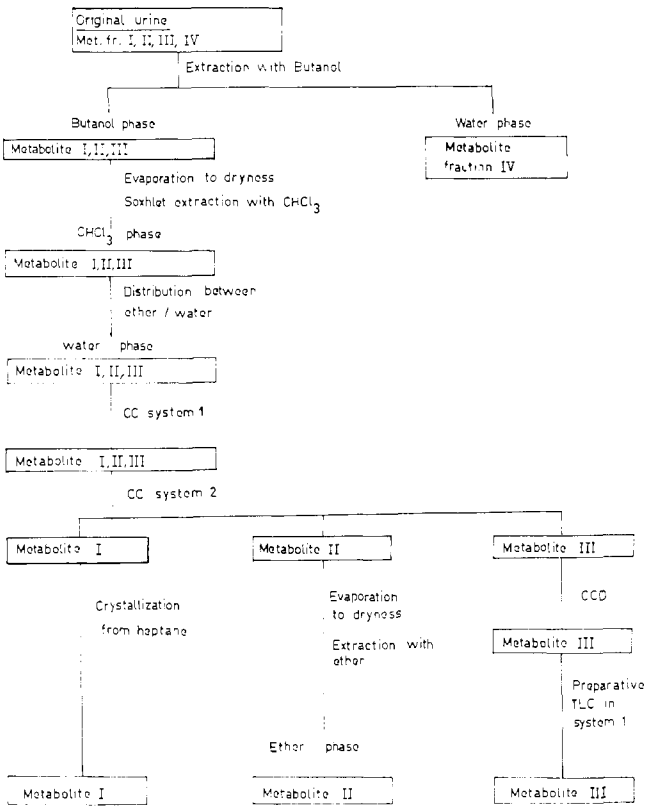


Figure 1. Isolation of urinary metabolites

of metabolite III was performed in the system ethylacetate and methanol–water (3:7 V./V.) with 80 steps.

Column chromatographic separation (CC) of the metabolites was achieved on a silica gel column (40 × 440 mm.) using chloroform–methanol (9:1) (system 1) and a gradient of 0–10% methanol in chloroform (system 2).

The metabolites of the urine were isolated according to the scheme outlined in Figure 1.

Measurements. Radioactivity was determined by liquid scintillation counting with the aid of a Tricarb liquid scintillation spectrometer, Model 3375 and Model 4312, Packard Instrument Corp. The quenching was corrected by internal standard method (scintillation cocktail 2 and 3) and by the channels ratio method (scintillation cocktail 1).

Three scintillation cocktails were used:

(1) For urine, extracts of feces and other solutions: dioxane 500 ml., glycolmonoethylether 200 ml., PPO 12 g., POPOP 0.6 g., naphthalene 60 g.
(2) For $^{14}CO_2$: toluene 666 ml., glycolmonoethylether 333 ml., PPO 5.5 g.
(3) For suspension of silica gel scrapped off from TLC plates: dioxane 500 ml., toluene 325 ml., PPO 5 g., POPOP 0.1 g., naphthalene 150 g., aerosil 37.7 g.

The radioactive zones on TLC were localized by a Scanner, Model 7201, Packard Instrument Corp.

The spectra were obtained with the aid of the following spectrometers:

IR spectra: Perkin Elmer Corp., Model 221, with NaCl prisms.
UV spectra: Beckman Instruments Inc., Model DK2-A.
NMR spectra: Varian Associates, Model HA-100.
Mass spectra: Consolidated Electrodynamics Corp., Model 21-110-B.

Table I. Balance Studies in Rats after Oral Application of ^{14}C-Diazinon

Excreta	Recovered Radioactivity[a] during 168 Hours (in per cent of dose applied)				Ethyl-^{14}C-Diazinon Male
	2-^{14}C-Diazinon				
	Males[b]		Females[c]		
Urine	80.0		68.9		65.4
Metabolite fraction 1		23		22	
Metabolite fraction 2		21		23	
Metabolite fraction 3		9		9	
Metabolite fraction 4		27		15	
Feces extract	16.0		23.5		17.5
Diazinon		1		1	
Metabolite fraction 1		6		10	
Metabolite fraction 2		5		6	
Metabolite fraction 3		2		3	
Metabolite fraction 4		2		3	
Feces residue	1.9		1.9		1.4
Expired air	<0.05		<0.05		5.6
Cage wash	0.4		0.3		0.3
Total recovery	98.3		94.6		90.2
Biological half life time	12 hours		12 hours		7 hours

[a] Dosage: 0.8 mg./rat. [b] Mean values of four animals. [c] Mean values of two animals.

Table II. Distribution and Dissipation of Radioactivity in Organs of the Male Rat after Subchronic Feeding of 2-^{14}C-Diazinon

(Dosage: 0.1 mg. per rat daily during 10 subsequent days)

Organs	Radioactive Content (in per cent of dose totally applied)				
	Time after Application in Days				
	0.25	1	2	5	8
Esophagus / Stomach	0.25	0.02	<0.01	<0.01	<0.01
Small intestine	0.65	<0.05	<0.05	<0.05	<0.05
Cecum, colon	0.76	<0.05	<0.05	<0.05	<0.05
Liver	0.16	<0.05	<0.05	<0.05	<0.05
Spleen	0.01	<0.01	<0.01	<0.01	<0.01
Pancreas	0.01	<0.01	<0.01	<0.01	<0.01
Kidneys	0.04	<0.01	<0.01	<0.01	<0.01
Lungs	0.02	<0.01	<0.01	<0.01	<0.01
Testis	0.02	<0.01	<0.01	<0.01	<0.01
Muscles	0.77	<0.30	<0.30	<0.30	<0.30
Fat	0.23	0.18	<0.10	<0.10	<0.10
Sum	2.92	0.20	...	...	...

RESULTS AND DISCUSSION

Balance Studies. The excretion of radioactivity after oral application of ring 2-^{14}C and side chain ^{14}C-labeled Diazinon to rats was followed in balance experiments. Concomitantly, the pattern of metabolites in the urine and the extracts of the feces was determined by TLC in system 1. The results are summarized in Table I.

Table I demonstrates the practically complete elimination of the insecticide and its metabolites. The rapidity of the metabolism is manifested by the time interval of 12 hours for the excretion of half the dose applied. The limits of detection of the isotope were reached 5 days after application. In all experiments the urine was found as the main route of excretion. The absence of radioactive CO_2 in the expired air after application of 2-^{14}C-Diazinon proves that no cleavage of the pyrimidine ring took place. This metabolic stability of the pyrimidine heterocycle was confirmed with 4-^{14}C-labeled Diazinon. An identical result was obtained with respect to both balance and pattern of metabolites. Only in the case of side chain labeled material a limited oxidation

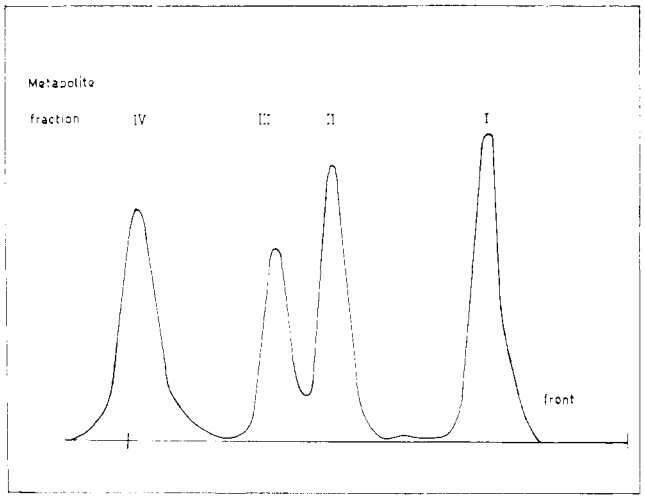


Figure 2. Separation of urinary metabolites of Diazinon by TLC in system 1

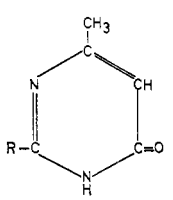
to $^{14}CO_2$ was observed. Whether this oxidation takes place before or after the pyrimidinyl phosphate bond has been cleaved was not investigated so that no indication on the possible existence of transient des-ethyl derivatives can be given.

The question of a specific accumulation of the insecticide or its metabolites in the essential organs of the rat was investigated after daily application of 2-^{14}C-Diazinon during ten days. After termination of the feeding period the dissipation curves of the isotope in the respective organs were determined. Table II presents the radioactivity found in each organ as mean values of two experiments.

The results of Table II clearly exclude any accumulation of the insecticide or its metabolites in the essential organs of the rat. No radioactivity was found later than 2 days after the end of the application period.

Structures of Metabolites. First analysis of the urine by TLC demonstrated the presence of 4 metabolite fractions as shown in Figure 2. The metabolite fractions 1, 2, and 3 were found to be homogenous, whereas fraction 4 contained a series of very polar substances. In addition to small amounts of unchanged Diazinon, metabolites 1, 2, 3, and fraction 4 were also present in the extracts of the feces. Diazoxon, as a labile and transient intermediate, was absent in the extracts of urine and feces when checked with TLC procedures, but without enzyme inhibition tests. Work on the elucidation of the structure of the most important metabolites was started by collecting 4.2 liters of urine from male rats fed with 2-^{14}C labeled Diazinon. Using the clean-up procedure described in Material and Methods (see Figure 1), metabolites 1, 2, and 3 were isolated in a pure form. Finally 48.3 mg. of metabolite 1, 84.3 mg. of metabolite 2, and 34.8 mg. of metabolite 3 were obtained.

The spectroscopic investigation of these metabolites by UV, IR, NMR and mass spectrum revealed that each of them contained the same heterocyclic moiety. This ring system has been identified as



by the following characteristic spectral data:

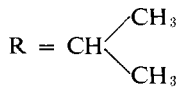
	IR		UV	NMR	
	C=O	C=C/C=N		HC=C—CH_3	
		(μ)	λ_{max}(mμ)	(τ)	
Metabolite 1	6.01	6.27/6.42	224/270	3.90	7.75
Metabolite 2	6.00	6.27/6.40	225/270	3.90	7.75
Metabolite 3	6.00	6.27/6.42	225/270	3.85	7.85

The metabolites 1, 2, and 3 differ only in the substituent R. The structures of these different fragments R are associated with the following characteristics:

Metabolite 1.

NMR: τ = 7.1 (septet, I = 7 Hz) = CH
τ = 8.7 (doublet, I = 7 Hz) = 2 equivalent CH_3

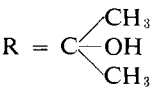
Mass-spectrum: molecular ion at m/e = 152



Metabolite 2.

IR: 2.88 μ = OH
NMR: τ = 8.45 (singlet) = 2 equivalent CH_3

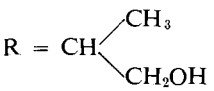
Mass-spectrum: molecular ion at m/e = 168



Metabolite 3.

IR: 2.95 μ = OH
NMR: τ = 5.65 – 6.20 (eight-line multiplet) = OCH_2
AB part of an $ABKX_3$-system
I_{AB} = 11 Hz
$I_{AK} \simeq I_{BK} \simeq 7$ Hz
= 6.75 (sextet, I $\simeq$ 7 Hz) = CH
= 8.60 (doublet, I = 7 Hz) = CH_3

Mass-spectrum: molecular ion at m/e = 168



The structures of the main metabolites 1 and 2 have been confirmed by spectroscopic comparison with synthetic reference material. [Metabolite 2 was synthesized in analogy to metabolite 1, *i.e.*, 2-isopropyl-4-methyl-6-hydroxypyrimidine (Gysin and Margot, 1958) using acetone cyanohydrin instead of isobutyronitril (Rüfenacht, 1968).]

The metabolites in the extracts of feces were identified by cochromatography with the corresponding ^{14}C-labeled metabolites isolated from the urine. Cochromatography with reference material was also used for the identification of the trace amounts of unchanged Diazinon.

Properties of Metabolites. In order to establish the degree of detoxication of the metabolites, the acute toxicity and the inhibitory activity on acetyl cholinesterase of the main metabolites 1 and 2 were determined. The results are presented in Table III.

Table III demonstrates that the expected, complete loss of the AChE inhibitory power and a drastic decrease of the acute toxicity of the insecticide during its metabolism had occurred. The AChE inhibition found with Diazinon is relatively high despite the fact that a several times crystallized preparation was used. This may be due to trace amounts of Diazoxon (having values in the range of 10^{-8}–$10^{-9}M$)

Table III. Biological and Toxicological Properties of the Main Metabolites

Compound	Acute Toxicity,[a] DL_{50}	Inhibition of AChE[b] ID_{50}
Diazinon	approx. 250 mg./kg.	$2.7 \times 10^{-5}M$
Metabolite 1 G 27'550	approx. 2700 mg./kg.	$>10^{-2}M$
Metabolite 2 GS 31'144	>5000 mg./kg.	$>10^{-2}M$

[a] Rat, oral application.
[b] Enzyme: soluble preparation from bovine red cells, (Serva, Heidelberg, Germany). Method: modified method of Michel (1949).

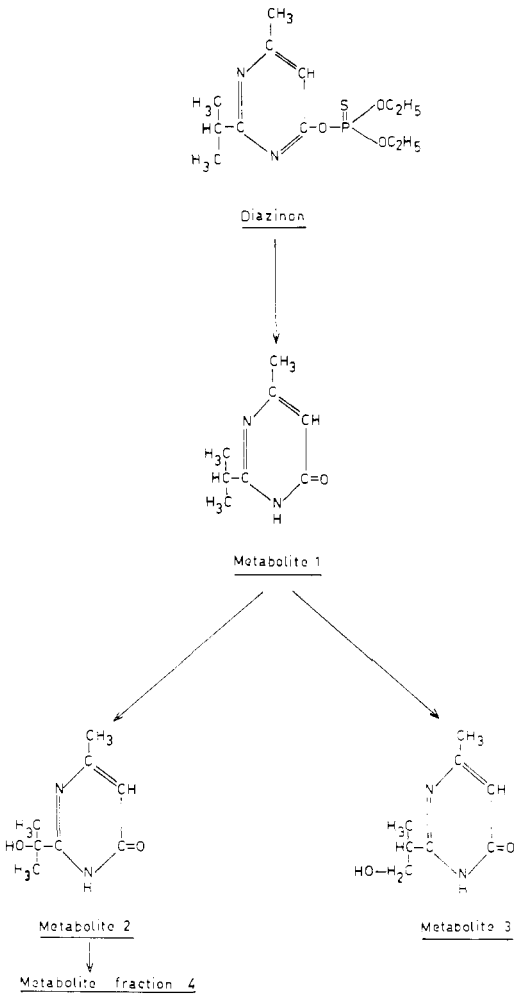


Figure 3. Elucidated steps of the metabolic pathway of the pyrimidine moiety of Diazinon in the rat

either still present or being formed during the incubation period of the test.

Pathway of Metabolic Degradation. The actual sequence of the particular degradation reactions was determined by following the fate of the isolated ^{14}C-labeled metabolites after their intravenous application. The excretion pattern is presented in Table IV.

Following the metabolism of the main metabolites after their intravenous application, a different further degradation of these compounds was observed as Table IV demonstrates. Metabolite 1 resulted in the same pattern of metabolites as observed with Diazinon itself. Metabolite 2 was excreted mainly unchanged and only 14% of the dose applied were

Table IV. Balance Studies and Pattern of Metabolites in the Male Rat after Intravenous Application of 2-^{14}C-labeled Metabolites 1, 2, and 3

Excreta and Metabolites Identified	Recovered Radioactivity[a] within 96 Hours (in per cent of the dose applied)		
	Metabolite 1[b]	Metabolite 2	Metabolite 3
Urine	74.7	77.6	87.1
Metabolite 1	26	0	0
Metabolite 2	17	65	0
Metabolite 3	13	0	87
Met. fract. 4	19	13	0
Feces extract	15.8	17.6	7.4
Metabolite 1	8	0	0
Metabolite 2	2	16	0
Metabolite 3	5	0	7
Met. fract. 4	1	1	0
Feces residue	1.2	1.1	3.0
Expired air	<0.05	<0.05	<0.05
Cage wash	0.3	0.4	0.6
Total recovery	92.0	96.7	98.1
Biological half life time	9 hours	9 hours	9 hours

[a] Dosage: Metabolite 1: 4 mg./rat. Metabolite 2: 8 mg./rat. Metabolite 3: 11 mg./rat.
[b] Mean values of two experiments.

transformed to metabolite fraction 4. In contrast to this further degradation of metabolites 1 and 2, metabolite 3 obviously is located on a side path of the metabolism since the molecule was excreted completely in an unchanged form.

The results obtained were summarized in Figure 3 in a scheme of pathways which were followed during the metabolic degradation of the pyrimidine ring system of the insecticide.

ACKNOWLEDGMENT

The skilled technical assistance of Miss V. Carenzi, Miss S. Kuenzli, Mrs. M. Lauber, Mrs. A. Stephan, and A. Walther with different parts of the work is gratefully acknowledged.

LITERATURE CITED

Bourne, J. R., Arthur, B. W., *J. Econ. Entomol.* **60,** 402–5 (1967).
Claborn, H. V., Mann, H. D., Younger, R. L., Radeleff, R. D., *J. Econ. Entomol.* **56,** 858–9 (1963).
Dahm, P. A., Koperky, A. E., Walther, C. B., *Toxicol. Appl. Pharmacol.* **4,** 683–96 (1962).
Derbyshire, J. C., Murphy, R. T., J. AGR. FOOD CHEM. **10,** 384–6 (1962).
Fukunaga, K., Presented on US-Japan Seminar on Experimental Approaches to Pesticide Metabolism, Degradation and Mode of Action, Aug. 16–19, Nikko, Japan (1967).
Fukunaga, K., Shishido, T., Fukami, J., 6th Int. Congr. Plant Protection, Vienna, Aug. 30–Sept. 6, 1967, Abstr., p. 202.
Gysin, H., Margot, A., J. AGR. FOOD CHEM. **6,** 900 (1958).
Harrison, D. L., Hastil, B. A., *New Zealand J. Agr. Res.* **9,** 1–7 (1965).
Kalberer, F., Rutschmann, J., *Helv. Chim. Acta* **44,** 1956 (1961).
Kaplanis, J. N., Louloudes, S. J., Roan, C. C., *Trans. Kansas Acad. Sci.* **65,** 70–75 (1962).
Krueger, H. R., O'Brien, R. D., Dauterman, W. C., *J. Econ. Entomol.* **53,** 25–31 (1960).
Matthysse, J. G., Gutenmann, W. H., Gigger, R., *J. Econ. Entomol.* **61,** 207–9 (1968).
Matthysse, J. G., Lisk, D., *J. Econ. Entomol.* **61,** 1394–8 (1968).
Michel, H. O., *J. Lab. Clin. Med.* **34,** 1564 (1949).
Millar, K. R., *New Zealand Vet. J.* **11,** 141–4 (1963).
Plapp, F. W., Casida, J. E., *J. Econ. Entomol.* **51,** 800 (1958).
Robbins, W. E., Hopkins, T. L., Eddy, G. W., J. AGR. FOOD CHEM. **5,** 509 (1957).
Rüfenacht, K., J. R. Geigy S. A., Agricultural Chemicals Research Department, private communication, 1968.
Vigne, J.-P., Chouteau, J., Tabau, R.-L., Rancien, P., Karamanian, A., *Bull. Acad. Vét. Fr.* **30,** 85 (1957).

Received for review August 11, 1969. Accepted December 4, 1969.