parenchyma cells as observed by Scheffer and Walker (*30*). This activation of anabolic processes does not occur when the resistant plant is pretreated with dinitrophenol, since the effectiveness of ATP-generating systems (*31*) is lost by this treatment; then the resistant plant becomes susceptible (*12*). We conclude that the suppression of pectic enzyme activity followed by an accelerated anabolic activity appears to be important defense reactions associated with the single-gene resistant character of tomato plants resistant to Fusarium wilt.

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PARASITICIDE METABOLISM

Residue and Metabolism of Radioactive 4-*tert*-Butyl-2-chlorophenyl Methyl Methylphosphoramidate Administered as a Single Oral Dose to Sheep

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Sheep were given single oral doses of 4-*tert*-butyl-2-chlorophenyl methyl methylphosphoramidate (Ruelene) parasiticide labeled with P^{32}; the metabolic fate was studied, and the residue in the tissues was determined for periods up to 21 days posttreatment. Ruelene and several hydrolysis products were found in the blood shortly after treatment, but by 2 days the Ruelene had decreased to a low level. Over 85% of the administered P^{32} was recovered in the excreta. The P^{32} in the urine, amounting to 75% of the dose, was primarily in the form of hydrolysis products of Ruelene. Some of the Ruelene was hydrolyzed completely to inorganic phosphate and retained in the animal tissues, in part as natural phosphate esters and in part as inorganic phosphate. Ruelene itself was not found in the tissues after 7 days.

RUELENE BRAND of 4-*tert*-butyl-2-chlorophenyl methyl methylphosphoramidate (registered trademark, The Dow Chemical Co., Midland, Mich.) possesses a rather broad spectrum of anthelmintic activity in cattle, sheep, and goats (*1, 3, 10, 11*), exhibits good systemic control of cattle grubs (*7, 9, 12*), and controls several types of ectoparasites (*7*). The purpose of this work was to examine the metabolic fate and tissue residue to Ruelene labeled with P^{32}, administered as single oral doses to sheep.

Experimental Methods

Two syntheses of Ruelene labeled with P^{32} (*8*) provided the material for the eight sheep experiments described. The first synthesis batch had a specific activity of 0.64 mc. per gram, and the second had a specific activity of 1.0 mc. per gram, on the dates of administration to the animals.

The animals were wethers of a Hampshire-Southdown cross, weighing 75 to 87 pounds when put on the test. They

were stanchioned in metabolism pens which permitted the separate collection of excreta. The radioactive chemical was weighed into gelatin capsules and administered with a balling gun. Four lambs were given 100 mg. of Ruelene per kg. of body weight, and four were given 200 mg. per kg. In addition, two animals were given 33.6 mg. per kg. of P^{32}-labeled phosphoric acid to provide a direct comparison with animals fed Ruelene under the experimental conditions.

The radioassays of tissue extracts, blood, urine, and fecal digests were made by liquid counting using a Geiger-Müller dip tube. Fat samples were counted as solids under an end-window Geiger-Müller tube. Paper chromatograms were first placed on film to produce radioautograms, then fed through a Geiger-Müller tube strip scanner.

Over-all Fate of P^{32} Activity

Figure 1 presents excretion data of one animal fed Ruelene and one fed inorganic phosphate. A major portion of the P^{32} from Ruelene appeared in the urine, primarily within the first 24 hours, and over 85% of the P^{32} in the original dose was recovered. In contrast, very little P^{32} from inorganic phosphate appeared in the urine, and less than one third of the original dose was recovered.

Figure 2 shows a comparison between the total P^{32} level in the blood of these same two animals. During the first 24 hours, the curves are very dissimilar, the feeding of Ruelene leading to a very early maximum. After the first day, the curves show a similar decline of activity.

Excretion and blood data indicate that Ruelene is rapidly absorbed, perhaps directly through the rumen wall, and rapidly eliminated. The nature of the elimination products will be discussed later.

The lower curve in Figure 2 is the chloroform-extractable P^{32} in the blood of the animal fed Ruelene. This includes Ruelene and one major metabolite of Ruelene, as will be shown later. Chloroform did not extract any detectable P^{32} activity from the blood of the inorganic phosphate-fed animal.

Essentially, the same results as given above were obtained with the other eight animals. When Ruelene was fed, 85 to 95% of the dose was recovered in the excreta, compared to 28% and 40% for the two inorganic phosphate-fed animals.

Tissue Residues

The chloroform-extractable P^{32} activity of tissues from animals fed 100 mg. of Ruelene per kg., an equivalent amount of inorganic phosphate, and

Table I. P^{32} Activity of Chloroform Extracts of Tissue

	Ruelene, 100 Mg./Kg.				H_3PO_4, 33.6 Mg./Kg.		Ruelene, 200 Mg./Kg.			
	Days									
Tissues	3	7	14	21	7	14	7	14[a]	14[a]	21
	P^{32} Activity, P.P.M. Ruelene									
Blood	0.1	0.3	...	0.0	0.1	0.0	...	0.0	...	...
Bile	0.1	0.3	...	0.0	0.1	0.0	...	0.0	...	...
Liver	1.5	1.0	0.1	0.1	2.5	6.0	0.1	0.0	0.1	0.0
Kidney	0.3	0.3	...	0.1	0.7	0.2	0.1	0.0	0.0	0.0
Spleen	0.1	0.2	0.0	0.0	0.6	0.4	0.1	0.1	0.1	0.0
Pancreas	4.5	0.2	0.1	0.1	5.2	5.6	0.1	0.1	4.4	0.1
Heart muscle	0.7	0.6	0.8	0.7	2.1	0.6	0.7	1.0	0.8	0.5
Tenderloin	0.1	0.1	0.1	0.2	0.4	0.4	0.2	0.3	0.2	0.4
Shoulder muscle	0.1	0.1	0.1	0.1	0.4	0.1	0.2	0.2	0.1	0.2
Rump muscle	0.1	0.1	0.1	0.1	0.4	0.2	0.2	0.3	0.3	0.3
Omental fat	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Perirenal fat	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Subcutaneous fat	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0

[a] Two different animals sacrificed at the same time.

Table II. Examination of Chloroform Extracts by Anticholinesterase Assay and Solvent Distribution

	Ruelene								H_3PO_4	
	Dose, Mg./Kg.									
	100		200		200		200		33.6	
	Days									
	21		7		14		21		14	
Tissues	A[a]	D[b]	A	D	A	D	A	D	A	D
Liver	...	0.3	0.0	0.2	0.0	...	0.0	...	...	0.2
Kidney	...	...	0.0	0.9	0.0	...	...	...	...	0.5
Pancreas	...	0.0	0.0	1.0	0.0	0.3	0.0	...	...	0.3
Heart muscle	0.0	0.1	0.0	0.2	0.0	0.3	0.0	0.3	...	0.5
Tenderloin	0.0	0.2	0.0	0.3	0.0	0.4	0.0	0.4	...	0.5
Shoulder	0.1	0.3	0.0	0.4	0.0	0.5	0.0	0.5	...	1.0
Rump	0.1	0.3	...	0.2	0.0	0.3	0.0	0.4	...	0.7

[a] Anticholinesterase values, p.p.m. of Ruelene.
[b] Distribution coefficient, acetonitrile-*n*-hexene. Ruelene gives 21.

Table III. Distribution of P^{32} Activity in Bone

	Ruelene				H_3PO_4	
	Dose, Mg./Kg.					
	100	100	200	200	33.6	33.6
	Days					
	7	14	14	21	7	14
	P^{32} Activity, p.p.m. Ruelene					
Ethylenediamine extractable	24	...	5.0	4.0	26	19
Residue	93	...	100	261	306	350
Total	117	110	105	265	332	369
	P^{32} Not Extracted, %					
	80	...	95	98	92	95

Table IV. Distribution of P^{32} on Incubation of Ruelene with Rumen Contents

	Distribution of P^{32}, %			
Time	Water layer	Solids	Chloroform layer	Recovered, %
10 min.	3.4	20	77	104
2 hr.	3.2	26	71	103
8 hr.	5.6	39	55	97
12 hr.	6.3	41	53	95
24 hr.	6.9	40	53	108
48 hr.	8.2	41	51	104

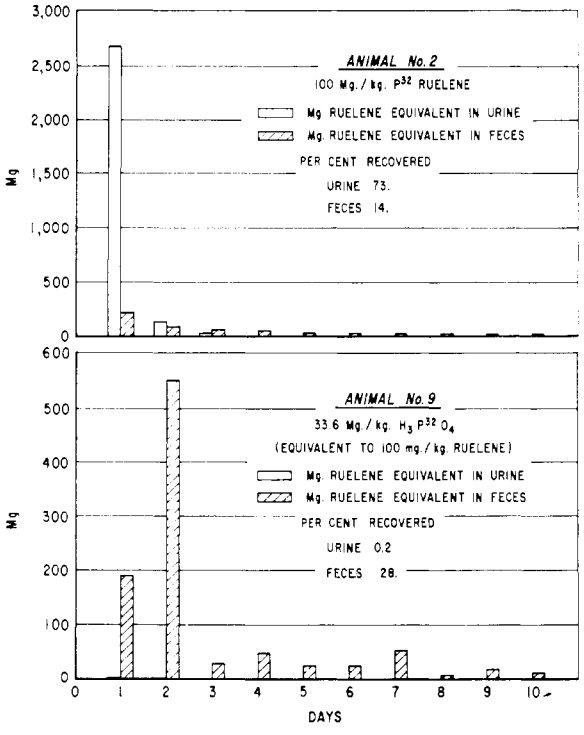

Figure 1. Excretion data of one animal fed Ruelene and one animal fed inorganic phosphate

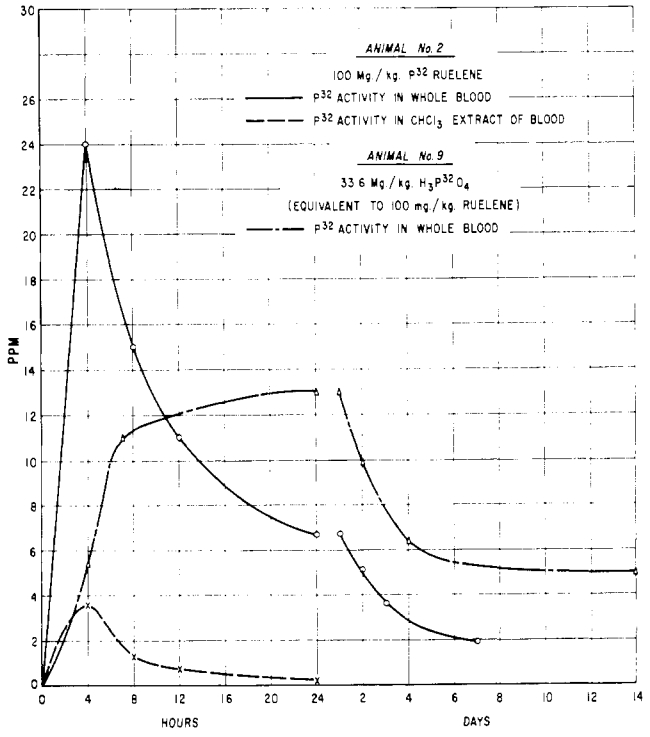

Figure 2. A comparison of the total P^{32} level in the blood of one animal fed Ruelene and one animal fed inorganic phosphate

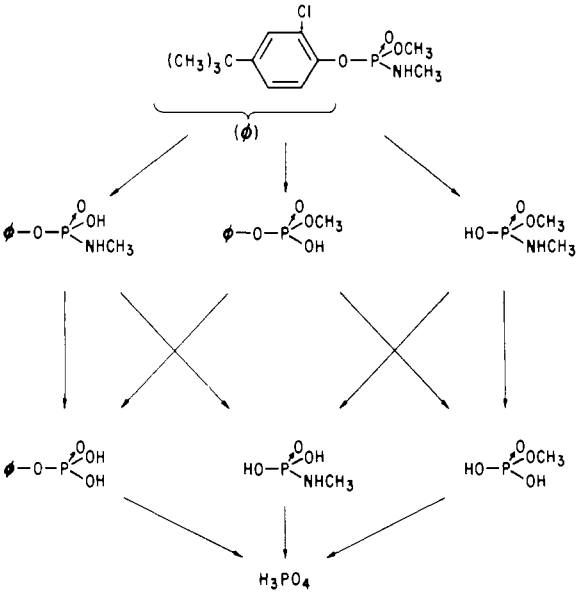

Figure 3. Six possible routes for the hydrolysis of Ruelene to inorganic phosphate

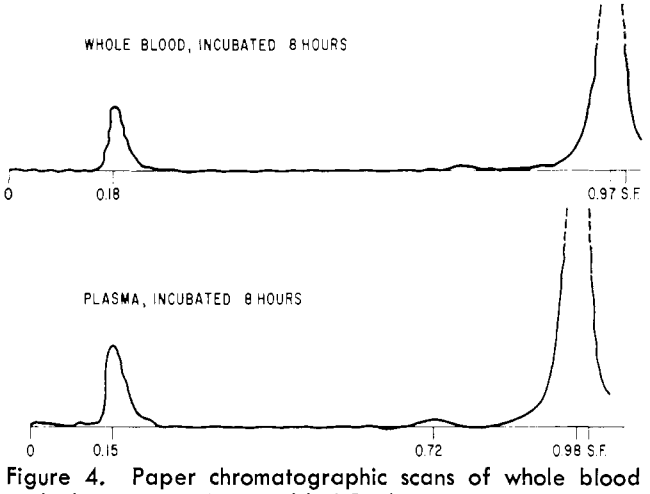

Figure 4. Paper chromatographic scans of whole blood and plasma, containing added Ruelene at a concentration of 100 p.p.m.

Known Compounds	R_f System IX
Ruelene ϕOP(O)OCH3(NHCH3)	0.98
ϕOP(O)OCH3(OH)	0.72
ϕOP(O)OH(NHCH3)	0.66
ϕOP(O)OH(OH)	0.22
HOP(O)OCH3(NHCH3)	0.18
HOP(O)OCH3(OH)	0.01
H3PO4	0.02

200 mg. of Ruelene per kg. are presented in Table I. The extraction procedure was a one-step chloroform extraction of a tissue homogenate, with a recovery of over 85% of labeled Ruelene added in vitro to tissue.

In contrast to most phosphorothioates, Ruelene does not enter, or at least does not remain in the body fat. This is very desirable from the point of view of lingering residues. However, some of the wet tissues do show measurable chloroform-extractable residues, even up to 21 days posttreatment. Notable in the slow decline of extractable P^{32} activity is the heart muscle tissue. Two of the values for pancreatic tissues appear to be too high in relation to the other six values. However, the feeding of inorganic phosphate also leads to high residual chloroform-extractable activity, presumably phospholipids, in heart muscle and pancreatic tissue, and in liver tissue as well. If, therefore, rather large amounts of the injested Ruelene were metabolized completely to inorganic phosphate, and evidence that this occurs is presented below, the chloroform-extractable P^{32} activity found in some tissues following the feeding of labeled Ruelene could be composed of natural nonpolar phosphate esters.

Two methods were used to examine these tissue extracts for Ruelene, and these data are presented in Table II. The A columns list results obtained when the chloroform extracts of tissue were submitted to anticholinesterase assay by the manometric assay method, using

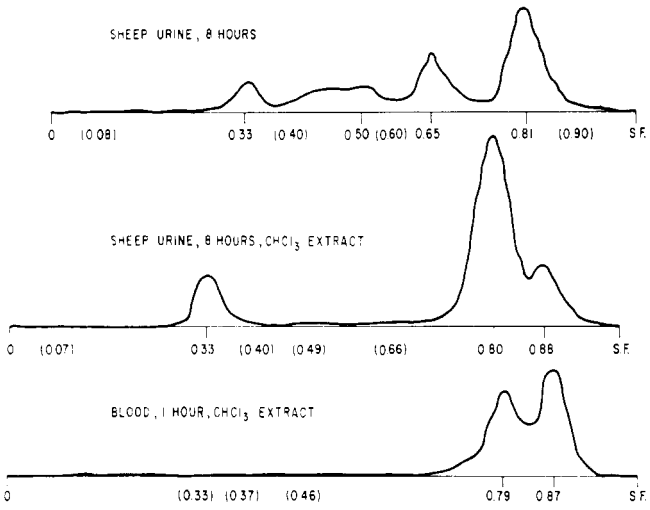

Figure 5. Scans of paper chromatograms of whole urine, a chloroform extract of the same urine, and also a chloroform extract of whole blood

Known Compounds	R_f, System VIII
Ruelene $\phi OP(O)OCH_3(NHCH_3)$	0.89
$\phi OP(O)OCH_3(OH)$	0.79
$HOP(O)OCH_3(NHCH_3)$	0.41
$\phi OP(O)OH(OH)$	0.49
$HOP(O)OCH_3(OH)$	0.13
H_3PO_4	0.05

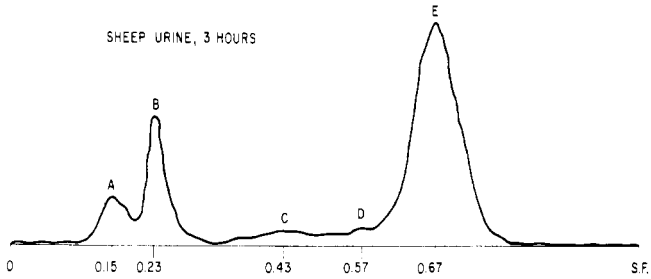

Figure 6. Paper chromatographic scan of sheep urine used in the quantitative study of urinary products

Known Compounds	R_f, System IX
Ruelene $\phi OP(O)OCH_3(NHCH_3)$	0.97
$\phi OP(O)OCH_3(OH)$	0.65
$\phi OP(O)OH(NHCH_3)$	0.64
$\phi OP(O)OH(OH)$	0.24
$HOP(O)OCH_3(NHCH_3)$	0.18
$HOP(O)OCH_3(OH)$	0.03
H_3PO_4	0.01

Time, Hours	Per Cent P^{32}				
	A	B	C	D	E
0–3	8	18	8	6	60
3–9	11	30	7	4	48
9–17	15	38	4	4	39
17–22	19	44	4	4	29

a fly-head enzyme preparation. This method was found to be sensitive to 0.05 p.p.m. of Ruelene added to tissue extracts. With two exceptions, no cholinesterase inhibition was observed. Of special note is the value of zero for the heart muscle extracts and pancreatic extract at 14 days following the feeding of 200 mg. of Ruelene per kg. This pancreatic extract contained a total P^{32} activity of 4.4 p.p.m., as shown in Table I, yet contained no detectable Ruelene.

The D columns of Table II list the distribution coefficients of the chloroform-extracted P^{32} activity distributed between *n*-hexane and acetonitrile. The chloroform extracts were evaporated, the residues taken up in *n*-hexane and acetonitrile, shaken in a separatory funnel, and the two layers were counted. Ruelene added to blank tissue extracts gave a distribution coefficient of 21. In all the tissue extracts, this value was much smaller, indicating that the P^{32} was present as compounds even less polar than Ruelene. The P^{32} activity in extracts from inorganic phosphate-fed animals was also nonpolar.

It is concluded that Ruelene is not residual in sheep tissues, even at 7 days after an oral dose of 200 mg. of Ruelene per kg.

Metabolic Fate of Ruelene

Table III presents data which indicate that part of the Ruelene is metabolized completely to inorganic phosphate. Using the ethylenediamine extraction method of Williams and Irvine (13), all the organic material of sections of rib bone was removed by extraction with 80% ethylenediamine in a Soxhlet extractor. This takes from 5 to 7 days, and only the purely inorganic bone matrix remains.

These values show that most of the residual P^{32} activity in bone is present as inorganic phosphate. This was confirmed by paper chromatography of a dilute acid solution of the bone residue.

A speculative estimate of the amount of Ruelene hydrolyzed by the animal to inorganic phosphate can be made using these data. The calculation is based on the assumption that the P^{32} activity found in bone tissue of both Ruelene, and phosphate-fed animals was directly proportional to the total body P^{32}-labeled inorganic phosphate, and the further assumption that the P^{32} activity in the feces of the two inorganic phosphate-fed animals represents phosphate not absorbed. Then, the per cent of the dose of Ruelene converted to inorganic phosphate is equal to:

$$\frac{\text{Total bone } P^{32}\text{, animal fed Ruelene}}{\text{Total bone } P^{32}\text{, animal fed phosphate}} \times \text{Per cent phosphate dose assimilated}$$

The calculations for the two pairs of comparable animals fed at the 100 mg. per kg., or equivalent dosage level, are as follows:

7-day animals: $110/369 \times 72 =$ 22% of dose of Ruelene

14-day animals: $117/332 \times 60 =$ 21% of dose of Ruelene

Thus, it appears that about one fifth of the Ruelene dose was metabolized completely to inorganic phosphate.

Figure 3 depicts the six possible routes of hydrolysis of Ruelene to inorganic phosphate. These intermediate compounds, with the exception of the methylamido dibasic acid, and the substituted phenylmethylamido acid, part of the time, were available to serve as chromatographic standards. Their position on paper chromatograms could be detected by the Hanes-Isherwood phosphomolybdate color test (5).

In studying the metabolic fate of a chemical given orally to sheep, the possibility that the chemical may be metabolized by the rumen microorganisms should be considered.

Table IV presents the results of one of several experiments using the artificial rumen technique. Ruelene labeled with P^{32} was incubated at 37° C., maintained at pH 7, and under CO_2, with fresh rumen contents from normal, untreated sheep. At the stated times, aliquots were removed, shaken with chloroform, and centrifuged. The three phases were separated and counted. The P^{32} activity in the chloroform extracts was examined by paper chromatography and found to be Ruelene only. The P^{32} activity in the solid and aqueous phases could not be identified by paper chromatography. Several attempts to extract the metabolites of Ruelene from other incubation mixtures with acetone or acetonitrile failed to yield any compound besides Ruelene. Thus, it appears that a major portion of the Ruelene dose may survive the rumen microorganisms.

Many attempts to demonstrate metabolism of Ruelene in vitro by tissue slices and homogenates failed. How-

ever, incubation of Ruelene with whole blood and with plasma gave definite evidence of hydrolysis, as shown in Figure 4. Ruelene was added to freshly drawn blood and plasma at a concentration of 100 p.p.m., incubated at 37° C., extracted with acetone, and the extract concentrated and chromatographed on Whatman No. 1 paper. A modification of the solvent system of Kaplanis and Robbins was used (*6*)—namely, 80% aqueous acetonitrile containing 2% of NH_4OH. R_f values of known hydrolysis compounds chromatographed at the same time are shown in Figure 4. The major product of these incubations appeared to be the dimethylamido phosphoric acid, and the secondary product may be the substituted phenylmethyl phosphoric acid. Washed red cells did not degrade added Ruelene. More degradation occurred when the plasma was adjusted to pH 9 than at the normal pH of 7.3. Very little degradation occurred at pH 5.

The best indication of the magnitude of the metabolic breakdown of Ruelene was obtained by paper chromatography of urine. Figure 5 presents scans of paper chromatograms of whole urine, a chloroform extract of the same urine, and also a chloroform extract of whole blood. Acid-washed Whatman No. 1 paper was developed descendingly with a modification of the solvent system of Ebel (*4*), consisting of 75% of aqueous isopropyl alcohol and 2% of NH_4OH. The R_f values in parentheses are for spots visible on the radioautograph, but not apparent in the scan.

The urine contained four major P^{32} containing components and four minor components, one of which, at R_f 0.90, was presumably Ruelene. The chloroform extraction of urine concentrated the small amount of Ruelene present, and also extracted to a varying degree some of the other components.

Using the heavy paper chromatographic technique suggested by Brownell *et al.* (*2*), milligram amounts of the front-running major components of whole urine and urine extracts were isolated, purified, and tentatively identified by their infrared spectra as a salt of the substituted phenylmethyl phosphoric acid, and Ruelene, respectively.

The chloroform extract of whole blood contained primarily these two compounds, as well as traces of three of the more polar metabolites.

Figure 6 contains data from a more quantitative study of the urinary products, wherein paper chromatograms of urine, collected at the stated time intervals following dosage, were sectioned and counted in a liquid scintillation instrument. These data show that the least polar metabolite, *E*, decreased in relative amount with time, in favor of two of the more polar components.

Attempts to isolate and identify the three major components of the first two collections of this urine were only partially successful. Component *E* was isolated in good purity and again appeared, by infrared spectroscopy, to be a salt of the substituted phenylmethyl phosphoric acid. Components *A* and *B* could not be sufficiently separated to permit complete identification by infrared, but both appeared to have lost the substituted phenyl moiety.

In summary, it appears that Ruelene parasiticide fed to sheep is rapidly absorbed, and also rapidly hydrolyzed, in part to partial hydrolysis products which are eliminated via urine, and in part to inorganic phosphate, which enters the normal body processes.

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INSECTICIDE METABOLISM IN PLANTS

Peroxidase and Ethylenediaminetetraacetic Acid–Ferrous Iron–Catalyzed Oxidation and Hydrolysis of Parathion

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MANY ORGANOPHOSPHATE ESTERS which have systemic insecticidal activity are oxidized within the plant. Such oxidations convert phosphorothionates to phosphates (*4, 5*) alkylthioalkyl groupings to their sulfinyl and sulfonyl derivatives (*1, 14*), and *N,N*-dimethylphosphoramides to *N*-methylphosphoramides through intermediate steps (*3, 10*). The rates of these oxidations vary greatly with the organophosphate, the plant species, and the physiological state of the plant (*14*). The mechanism of this oxidation in plants is not known. Since plants can oxidize parathion (*O,O*-diethyl *p*-nitrophenyl phosphorothionate) to para-oxon (*O,O*-diethyl *p*-nitrophenyl phosphate) (*5*), this phosphorothionate was selected as the substrate for the present studies.

Mason (*13*) has presented evidence in support of the view that peroxidase, functioning as an oxidase, activates molecular oxygen toward aromatic substrates. The hydroxylating system of Udenfriend *et al.* (*18*) involving ascorbic acid, ferrous iron, and (ethylenedinitrilo)-tetraacetic acid (ethylenediaminetetraacetic acid, EDTA acid) functions in a somewhat similar manner. The EDTA-Fe^{+2} complex is a good synthetic model for peroxidase. In the presence of ascorbic acid, peroxidase is inactive, but