considered toxic to livestock, which is in close agreement with the work conducted in Georgia (*2*).

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METABOLISM OF INSECTICIDES

Metabolism and Excretion of Phosphorus-32–Labeled Diazinon in a Cow

WILLIAM E. ROBBINS, THEODORE L. HOPKINS, and GAINES W. EDDY

Entomology Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Corvallis, Ore.

Phosphorus-32–labeled Diazinon, administered orally to a cow at 20 mg. per kg., is rapidly metabolized and excreted. Only low levels of unchanged toxicant were found in blood and milk samples. About 74% of the dose, excreted as polar degradation products, was accounted for in the urine 36 hours after treatment.

DIAZINON, the organic phosphorus compound, *O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidyl) phosphorothioate, has been evaluated against a wide variety of insects affecting plants and domestic animals (*5*). It is an effective toxicant in baits (*4*) and, by residual application, in the control of houseflies in dairy barns (*7*).

McGregor and coworkers (*11*) have demonstrated that Diazinon is a promising chemotherapeutic agent when administered orally or subcutaneously to cattle parasitized by cattle grubs, *Hypoderma lineatum* (De Vill.). It is also effective when applied externally to cattle as a back treatment (*16*).

More recently the distribution and excretion of Diazinon in guinea pigs (*9*) and its penetration and excretion by the American cockroach (*1*) have been studied with the aid of phosphorus-32–labeled Diazinon and paper chromatographic analysis.

To gain a better understanding of its action as a mammalian systemic insecticide and to investigate the nature of its metabolic breakdown products in the bovine body, phosphorus-32 Diazinon was administered orally to a Hereford cow and, at intervals thereafter, samples of blood, milk, urine, and feces were assayed radiometrically and/or chromatographically.

Materials and Methods

Experimental Animal. The animal employed in this study was a lactating Hereford cow. At the time of administration of the labeled compound the cow weighed 267.6 kg.

Radioactive Compound. The phosphorus-32–labeled Diazinon was synthesized by Louloudes and associates (*10*). The radioactive compound was diluted with nonradioactive Diazinon (99.2% pure, from Geigy Agricultural Chemists Research Laboratory, Bayonne, N. J.) and, at the time of administration, the diluted material had an observable specific activity of 5.18×10^8 counts per minute per gram (mean of three determinations). The radiochemical purity of the phosphorus-32 Diazinon was found to be greater than 99% when assessed by several paper chromatographic methods of analyses.

Administration of Radioactive Compound. The labeled Diazinon was administered orally in a No. 11 gelatin capsule (0.5 ounce) by use of a balling gun. The dosage was 20 mg. per kg. (5.232 grams for the 267.6-kg. animal).

Sample Collection. The animal was held in a metal chute modified for tracer studies. Food and water were supplied throughout the experimental period.

Urine and feces were collected manually, with considerable care to prevent cross contamination. Near-quantitative collections were made for the 36-hour post-treatment interval. Subsequent to this period, samples were taken only at the various times indicated in the figures and text.

Blood samples, 10 ml., were taken from the jugular vein at each sampling; sodium citrate was used as the anticoagulant.

The animal was milked manually. Previous to milking, the udder was carefully washed and checked with a survey meter to avoid contamination of the sample. An attempt was made to remove all available milk at each sampling.

Following collection and radiometric analyses, formalin was added to the blood, milk, and urine as a preservative and the samples were held under refrigeration until extractions and/or chromatographic analyses could be performed.

Radiometric Measurements and Sample Preparation. Radiometric analyses were made with a conventional scaler and a windowless gas-flow proportional counter. All samples were prepared in duplicate, and the mean corrected counting rate was used in computing the radioactivity. The samples were counted for a sufficient time to attain a maximum standard error of $\pm 5\%$ (*2*). The counting rates were corrected for decay and expressed as microgram- or milligram-equivalents of Diazinon.

Samples were prepared by plating the materials to be analyzed on aluminum or stainless steel planchets. To ensure even distribution of the sample, disks of tissue paper, 2 cm. in diameter, were attached to the bottom of the planchets. Care was taken to keep the sample thickness below the level of appreciable self-absorption. To prevent loss of radioactivity during drying and to avoid contamination of the windowless counter, 2 to 3 mg. of Carbowax 400 (polyethylene glycol) was added to the samples.

The blood, urine, and milk, and extracts of these fluids were assayed as plated measured aliquots, as previously described. The feces were treated in the following manner:

After thorough mixing with an electric stirrer, 2-gram samples (wet weight)

Table I. R_f Values for Diazinon and Related Compounds

Compound	Mean R_f Values at 23–25° C. Silicone reversed-phase	Carbowax 4000–petroleum ether	Pyridine–ammonium hydroxide
Diazinon	0.07	0.90	0.81
Diethyl phosphorothioic	0.92	0.03	0.63
Diethyl phosphoric acid	0.98	0.05	0.44

were weighed into No. 13 gelatine capsules and placed in calibrated blood-sugar tubes. The samples were wet-digested with concentrated nitric, sulfuric acid, and 30% hydrogen peroxide. The digest was made up to a total volume of 10 ml., and aliquots were plated on stainless steel planchets, heat-dried, and assayed. When control fecal samples, to which 5 to 50 γ per gram of labeled Diazinon had been added, were treated in the same manner, a mean recovery of 97% was obtained (range 93 to 102%).

Methods of Analysis. The chromatographic systems used in this study were:

1. Silicone reversed-phase (*14*). Whatman No. 1 paper was coated with a 5% solution (v./v.) of the stationary phase, Dow Corning Silicone 550, in acetone. The developing solvent was the upper phase from a mixture of 4 parts of ethyl alcohol, 4 parts of chloroform, and 2 parts of water. Chromatograms were developed by the ascending method.
2. Carbowax 4000–petroleum ether (*9*). Strips of Whatman No. 1 paper were coated with the stationary phase by passing them through a 5% (w./v.) chloroform solution of Carbowax 4000. The mobile phase consisted of 96.5 parts of petroleum ether (30° to 60° C.) to 3.5 parts of glacial acetic acid (v./v.). Ascending development was employed.
3. Pyridine–ammonium hydroxide. The developing solvent in this method consisted of 8 parts of pyridine to 2 parts of ammonium hydroxide (specific gravity 0.90). The solutions to be analyzed were applied to strips of Whatman No. 1 paper and these were allowed to equilibrate in a chamber containing the developing solvent for about 1 hour. The sides of the chamber were lined with filter paper immersed in the solvent to ensure saturation of the atmosphere. After equilibration, the strips were lowered into the solvent and the chromatogram was developed by the ascending method.

The R_f values for Diazinon and related compounds, determined by the above methods, are reported in Table I. These were determined colorimetrically and/or radiometrically. Diazinon was detected with an acid permanganate solution (*9*). Diethyl phosphoric acid, diethyl phosphorothioic acid, and the metabolic products present in the urine gave color development by the method of Hanes and Isherwood (*6*). Radiometric analyses were performed by cutting the developed chromatograms into 0.5- or 1-cm. strips, which were assayed in a windowless proportional counter.

A 20- to 30-cm. development was employed with all systems. The silicone–reversed-phase system was found to be best suited for the analysis of blood and milk extracts as no appreciable "tailing" or R_f shifts were caused by the interfering substances present in these extracts. The Carbowax–petroleum ether system, which reversed the order of the R_f values, served as an additional check on the results obtained by the silicone–reversed-phase system. The pyridine–ammonium hydroxide system was employed in the separation and identification of the metabolic products present in the urine, which remained near the point of application or ran near the front by the Carbowax–petroleum ether and silicone–reversed-phase systems, respectively.

The partition of blood radioactivity into hexane was determined in the following manner:

Equal volumes of blood and hexane were thoroughly shaken in a stoppered, graduated centrifuge tube and centrifuged to ensure separation. Duplicate samples of the hexane layer were plated and assayed radiometrically and the per cent of radioactivity partitioning into the hexane layer was calculated from the corrected counting rates. When samples of control blood to which 1 and 3 γ of phosphorus-32 Diazinon per ml. had been added were treated in the same manner, greater than 98% of the Diazinon was found to partition into the hexane layer.

For chromatographic analysis, the blood samples were pooled because of the small amounts of radioactivity present and extracted two additional times with equal volumes of hexane to effect a more complete removal of the organo-soluble compound(s). The composite of the three hexane extracts was then extracted with three equal volumes of acetonitrile by the method of Jones and Riddick (*8*). Radiometric analysis of the hexane layer following cleanup demonstrated near quantitative removal of radioactivity by the acetonitrile. The hexane extractions of the milk samples and the acetonitrile cleanup of the hexane extracts were as follows:

One hundred milliliters of ethyl alcohol was added to 100 ml. of milk and the mixture was extracted with three 200-ml. aliquots of hexane. The hexane extracts were pooled, concentrated, assayed radiometrically, and extracted with three equal volumes of acetonitrile saturated with hexane. The hexane layer was assayed radiometrically to determine the percentage of radioactivity extracted. When control milk samples, to which 1, 2,

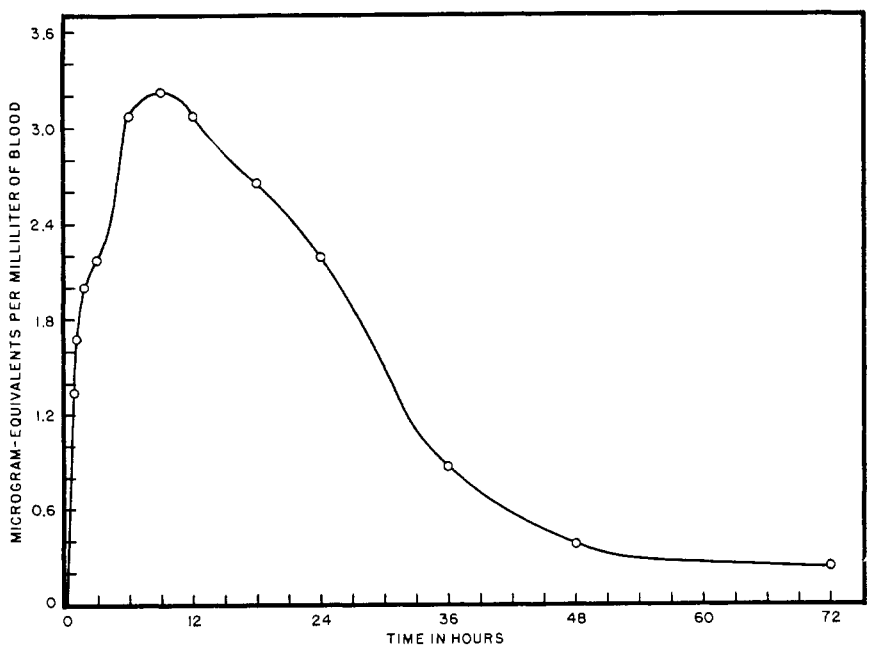

Figure 1. Radioactivity in blood of cow following oral administration of phosphorus-32 Diazinon

Table II. Partition of Blood-Radioactivity into an Equal Volume of Hexane.

Sample, Hr.	γ Equivalents /Ml.	Partitioned into Hexane %	Partitioned into Hexane γ Equivalents /ml. blood
2/3	1.34	4.9	0.07
1	1.66	5.6	0.09
2	2.04	5.5	0.11
3	2.16	6.7	0.14
6	3.08	6.2	0.19
9	3.21	7.3	0.23
12	3.08	9.3	0.29
18	2.66	7.7	0.20
24	2.19	5.0	0.11
36	0.87	3.9	0.03

and 3 γ of phosphorus-32 Diazinon per ml. had been added, were extracted as previously described, 98.8% of the added Diazinon was extracted by hexane and 97.3% partitioned into the acetonitrile (means of three determinations).

Aliquots of the 36-hour composite urine were concentrated by passing a gentle stream of air over the sample. The concentrate was acidified with dilute acid and extracted repeatedly with acetone. The acetone extracts were analyzed chromatographically by the pyridine–ammonium hydroxide system. To determine the percentage of unchanged Diazinon present, aliquots of whole urine were assayed by the silicone–reversed-phase system.

The bioassay method used to assess the toxicity of the radioactive compound(s) present in the milk was the feeding technique of Sun and Sun (*17*), with a modification; 3- to 4-day old female houseflies of a normal strain were employed, 50 flies in each determination, and the sample to be evaluated was placed in a Petri dish containing cotton.

Results

Blood. The amounts of radioactive compounds found in the blood are shown in Figure 1. No significant quantities of radioactivity were detected in the 96- or 120-hour samples.

When the blood was extracted with an equal volume of hexane, as previously described, only a small percentage of the radioactivity present behaved like Diazinon, Table II. The maximum concentration of partitioned radioactivity (0.29-γ equivalents per ml.) did not coincide with the peak of radioactivity in the blood, but was found in the 12-hour sample.

Chromatographic analysis of the composite extract by the silicone–reversed-phase system demonstrated that only 18.1% (mean of four chromatograms) of the radioactivity ran with the R_f of Diazinon. The remainder formed an elongated spot with a mean R_f of 0.67. By the Carbowax–petroleum ether system the radioactivity formed a single large spot at R_f 0.70, the top of which overlapped the R_f of Diazinon.

Following hexane extraction, the proteins were precipitated from the blood by the addition of an excess (four to five volumes) of acetone. After filtration, the acetone was removed from the filtrate with a gentle stream of air and the aqueous portion concentrated. When the aqueous concentrate was analyzed by the silicone–reversed-phase system all the radioactivity ran near the front (R_f 0.9 to 1.0), and coincided with the R_f of diethyl phosphoric and diethyl phosphorothioic acid.

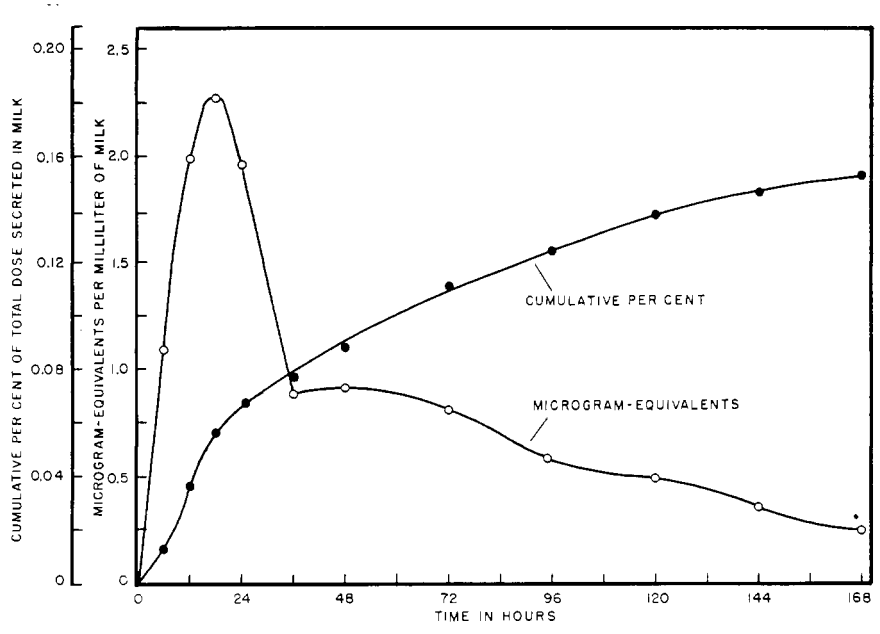

Figure 2. Radioactivity in milk of cow following oral administration of phosphorus-32 Diazinon

Milk. The results of radioassay of the milk samples are given in Figure 2.

When the milk samples were extracted with hexane, as previously described, only one fourth to one third of the radioactivity present in the 6- through 24-hour samples behaved like unchanged Diazinon (Table III). There followed a sharp decline in the percentages of radioactivity extracted in the 36- and 48-hour samples, with only trace amounts found in the extract of the 72-hour milk sample. No radioactivity was detected in the

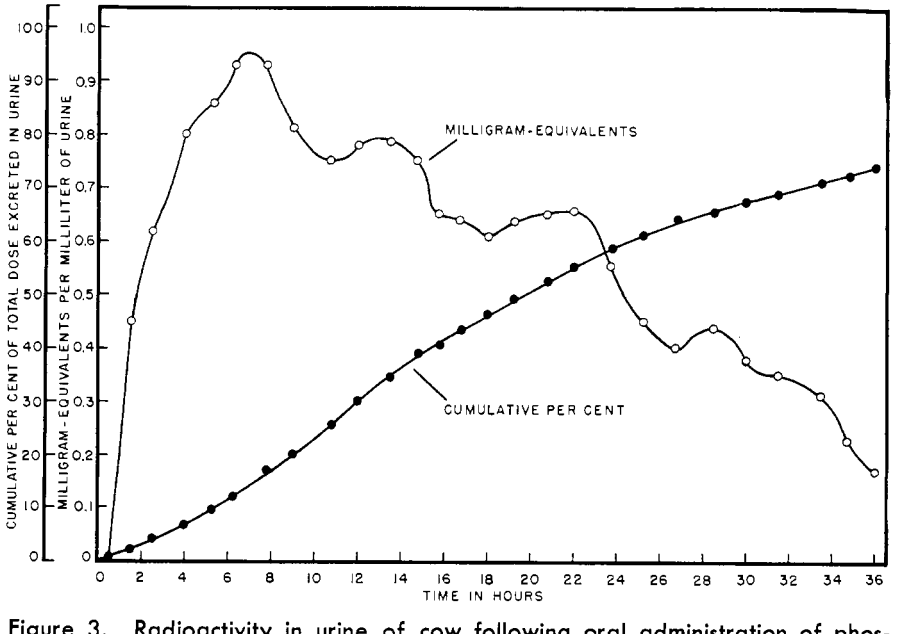

Figure 3. Radioactivity in urine of cow following oral administration of phosphorus-32 Diazinon

hexane extracts of the 96- or 120-hour milk samples.

Following acetonitrile cleanup the extracted radioactivity was analyzed by the silicone–reversed-phase system. By this method, 32, 63, 70.9, and 57.5% of the radioactivity extracted from the 6-, 12-, 18-, and 24-hour samples ran with R_f of Diazinon, giving concentrations of 0.09, 0.40, 0.56, and 0.25 γ of Diazinon per ml., respectively (Table III). In all these samples, the remainder of the extracted radioactivity ran near the front (R_f 0.9 to 1.0). The levels of radioactivity present in the hexane extracts of the 36- to 72-hour samples were too low to permit chromatographic analysis.

These findings were further confirmed by the results of bioassay of the milk samples (Table IV). These data indicate that the radioactive compound(s) present in the milk are considerably less toxic to houseflies than a radioactive equivalent of the parent compound when assayed by the feeding technique.

Urine. Diazinon or its degradation products were rapidly excreted in the urine with 74% of the total dose accounted for in 36 hours (Figure 3). Samples taken at 48 and 72 hours contained 0.1- and 0.002-mg. equivalents, respectively.

Attempts to extract the radioactivity from portions of the 36-hour composite urine, acidified or neutral, with several organic solvents—i.e., chloroform, carbon tetrachloride, hexane, diethyl ether, petroleum ether, and benzene—were unsuccessful. To determine if any of the administered Diazinon had been completely metabolized to inorganic phosphorus during this period, samples of the composite urine were treated with "magnesia mixture." Radiometric assay demonstrated that only trace amounts of radioactivity behaved as inorganic phosphorus.

When the 36-hour composite urine was analyzed by the silicone–reversed-phase system, nearly all of the radioactivity ran near the front (R_f 0.9 to 1.0), and only about 0.2% behaved as unchanged Diazinon. When these radioactive compounds, removed from the urine as previously described, were analyzed by the pyridine–ammonium hydroxide system, it was found that 50.5% ran with the R_f of diethyl phosphorothioic acid and 44.8% ran with the R_f of diethyl phosphoric acid (means of four runs) (Figure 4). Trace amounts ran with the R_f of Diazinon and the remainder of radioactivity remained near the point of application.

Feces. Radioassay data of the wet-ashed fecal samples collected to 48 hours are shown in Figure 5. The 72-hour sample contained 1.2 γ equivalents per gram, and only trace amounts of radioactivity were found in the 96-hour fecal sample. No activity was detected in the 120- or 144-hour samples.

Table III. Hexane Extraction of Milk Samples and Chromatographic Analysis of Extracted Radioactivity Following Acetonitrile Cleanup

Sample, Hr.	γ Equivalents/ Ml. of Milk	Hexane Extraction[a]		Chromatographic Analysis[b]	
		Extracted, %	γ Equivalents ml. of milk	Diazinon, %	γ-Diazinon/ ml. of milk
6	1.09	25.6	0.28	32.0	0.09
12	1.99	32.2	0.64	63.0	0.40
18	2.27	34.8	0.79	70.9	0.56
24	1.96	22.4	0.44	57.5	0.25
36	0.88	9.1	0.08	[c]	—
48	0.91	2.2	0.02	[c]	—
72	0.81	<1[d]	<0.008[d]	[c]	—
96	0.58	0	0	—	—
120	0.49	0	0	—	—

[a] Means of 2 extractions.
[b] Chromatographed by silicone–reversed-phase system; means of 2 to 4 chromatograms.
[c] Quantity of interfering substance too great to permit chromatographic analysis of low levels of radioactivity present.
[d] Counting rates too low for significance.

The feces were bioassayed by infesting portions of the samples with approximately 50 newly-hatched horn fly and housefly larvae. Following the infestation of the 1- through 72-hour samples with horn fly larvae, development and emergence occurred only in the 1- and the 72-hour samples. The 48- and 72-hour samples were also infested with housefly larvae and again emergence occurred in the 72- but not in the 48-hour sample. These data indicate the presence of a toxic compound(s) in the 6- through 48-hour fecal samples.

Table IV. Bioassay of Milk Samples by Feeding Method Employing 3- to 4-Day Old Female Houseflies

Milk Sample		Mortality, %		
Hour	γ Equiv./ml.	24 hr.	48 hr.	72 hr.
Milk from Treated Cow				
6	1.09	0	6	22
12	1.99	4	10	22
18	2.27	0	14	30
24	1.96	2	6	16
36	0.88	0	0	14
48	0.91	2	4	10
72	0.81	6	8	14
Control		0	0	14
P^{32} Diazinon Added to Control Milk				
	γ/ml.			
	0.25	2	10	24
	0.50	0	16	36
	1.00	6	40	68
	2.00	26	64	84
	3.00	68	90	100

Discussion

When the metabolic fate of Diazinon and Bayer L 13/59 (*O,O*-dimethyl-2,2,2-trichloro - 1 - hydroxyethylphosphonate)

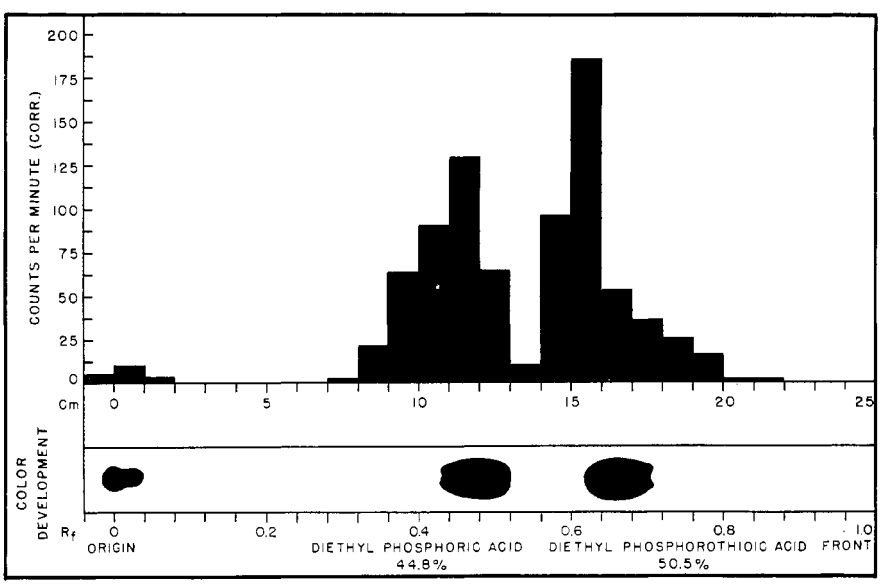

Figure 4. Paper chromatogram of phosphorus-32–labeled urine metabolites by pyridine–ammonium hydroxide system

Color development by Hanes-Isherwood (1949) method. Percentages are means of four runs

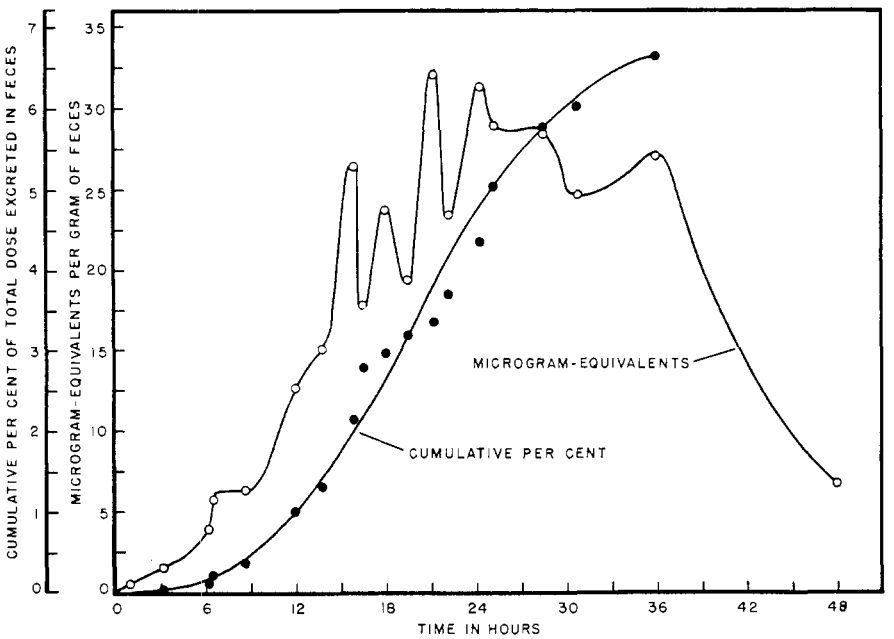

Figure 5. Radioactivity in feces of cow following oral administration of phosphorus-32 Diazinon

(*15*), also a potential chemotherapeutic agent, are compared, several similarities and differences are notable.

Both compounds appear to be efficiently absorbed from the digestive tract following oral administration, as evidenced by the prompt appearance of radioactivity in the blood and/or the relative percentage of the administered dose accounted for in the urine and in the feces. The absorption of Bayer L 13/59, however, appears to proceed more rapidly.

Higher levels of radioactivity and the unmetabolized toxicant were attained in the blood of the animal treated with phosphorus-32–L 13/59. Instead of the abrupt early maximum found in the 2-hour sample and immediate decrease in the concentration of radioactivity present in the blood of the Bayer L 13/59 treated animal, a plateau was maintained throughout the 24-hour period following the administration of phosphorus-32 Diazinon with the highest concentration found to be present in the 9-hour sample.

Bayer L 13/59 is metabolized and excreted much more rapidly than is Diazinon. At the end of 12 hours after treatment, about 66% of the radioactivity of a 25 mg. per kg. dose of Bayer L 13/59 could be accounted for in the urine; when Diazinon was administered at the rate of 20 mg. per kg. only about 30% was excreted via the urine in the same interval of time.

The major portion of organosoluble radioactivity present in the blood of the Diazinon-treated cow appeared to be a more polar metabolic intermediate(s), that ran with a different R_f, than Diazinon by both the silicone–reversed-phase and Carbowax–petroleum ether systems. That the unknown metabolite(s) was not diethyl phosphorothioic and/or diethyl phosphoric acid was confirmed by the latter system, as these two compounds remain near the point of application (R_f 0.03 and 0.05) when chromatographed by this system (Table I).

The phosphorus-32–labeled end products of Diazinon metabolism in the cow were found to be diethyl phosphorothioic and diethyl phosphoric acids. The former compound is formed by the enzymic hydrolysis of Diazinon; the latter could be formed either by the enzymic oxidation of the parent thionophosphate to its phosphate, *O,O*-diethyl-2-(2-isopropyl-6-methyl-4-pyrimidyl) phosphate, and the subsequent hydrolysis of this compound and/or the oxidation of diethyl phosphorothioic acid after its formation by the hydrolysis of Diazinon. It is not known if any of the diethyl phosphoric acid produced by the cow is formed by oxidation previous to hydrolysis; however, the presence of the more polar, unknown compound(s) in the blood following administration of phosphorus-32 Diazinon indicates the occurrence of intermediate metabolic reaction(s) prior to hydrolysis. The enzymic oxidative process has been reported to occur in both insects and mammals with other thionophosphates as the substrate, with the production of a more potent anticholinesterase agent (*3*, *12*, *13*). Afifi and Roan (*1*) report that the foregut of the American cockroach appears to metabolize Diazinon to a water-soluble metabolite with greatly increased anticholinesterase activity.

Although low levels of unchanged Diazinon were found in the 6- through 24-hour milk samples, the total amount of unchanged toxicant secreted in the milk during this period was only 631 γ, or about 0.01% of the administered dose. The concentration of toxicant present in the various milk samples appeared to be in proportion to the quantity of hexane-soluble compound(s) present in the corresponding and/or previous blood sample and no tendency toward storage and subsequent secretion was noted. The unknown compound(s) found to be present in the blood was not detected in the hexane extract of the milk.

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