Fate of Temik–Carbon-14 in Lactating Cows During a 14-Day Feeding Period

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Increasing the number of days, up to 14, that cows were administered Temik [2-methyl-2(methylthio-C^{14}) propionaldehyde *O*-(methylcarbamoyl) oxime] at levels of 0.12, 0.6, and 1.2 p.p.m. in the diet did not alter the magnitude and nature of residues eliminated daily in the milk, urine, and feces. Parts per million total Temik equivalents in the milk were approximately $^1/_{100}$ that level of insecticide in the feed. About 15% of the radioactive residues in the milk was Temik sulfone and about 4% was Temik sulfoxide. The remaining was hydrolytic products and compounds of unknown identity. Percentages of the doses eliminated in the milk, urine, and feces were 1, 92, and 3, respectively. Total Temik equivalents in the liver were 29, 123, and 164 p.p.b. for the three treatment rates, respectively, when the animals were slaughtered 18 hours after the last treatment. Twenty-six other tissue samples contained either much lower quantities of residues or none at all.

An earlier report showed that when Temik [2-methyl-2-(methylthio) propionaldehyde *O*-(methylcarbamoyl) oxime], also known as an aldicarb, was administered to a lactating cow as a single oral dose, approximately 85% of the dose was eliminated from the body within 24 hours (Dorough and Ivie, 1968). Temik equivalents in the milk were low, maximum of 62 p.p.b., and no Temik *per se* was detected. The nature of the metabolites was similar to that reported for other animals and for plants. The data indicated that the rapid excretion of Temik, primarily in the urine, by dairy animals would prevent the accumulation of residues in milk and tissues to appreciable levels. However, this could not be stated with certainty because continuous feeding of Temik could alter its metabolic fate, possibly resulting in higher residues and/or ones different chemically from those found after a single treatment.

The need for long-term, continuous feeding studies stems largely from the behavior of the chlorinated hydrocarbon pesticides. Certain of these toxicants appear in milk even when very low levels are in the cow's feed. Furthermore, several weeks, and often several months, of feeding are necessary before the concentration of residues in the milk reaches a plateau. For example, heptachlor epoxide residues appeared to be still increasing at the end of a 35-day feeding period (Williams *et al.*, 1964). With dieldrin, endrin, lindane, and DDT, the concentration of each residue in milk had reached a maximum at various times during the feeding period. The nature of these chemicals continues to influence the investigational format of other groups of pesticides, such as the carbamates, despite the fact that these newer products are different chemically, biologically, and metabolically. Obviously, a format based on the chlorinated hydrocarbons would not necessarily be appropriate for all other insecticides. In addition to establishing the fate of Temik in cows when administered over a long period of time and correlating dosage rates to residue levels in meat and milk, the present investigation should be useful in determining experimental parameters most desirable for future studies using similar compounds.

The concentration of Temik and/or its metabolites in mature forage crops treated with Temik has not been reported. However, sufficient work has been done to indicate the type of metabolites that must be considered. Temik is readily oxidized in cotton to its sulfoxide which is then slowly metabolized to the sulfone (Coppedge *et al.*, 1967; Metcalf *et al.*, 1966). When applied to the soil, maximum uptake of Temik-S^{35} by cotton plants occurred within 2 weeks, with no indication of significant uptake thereafter. After 6 weeks, Temik sulfoxide and Temik sulfone were present in mature leaves in about equal quantities. No Temik was detected. These data demonstrate that the residues likely to be present in feeds would be Temik sulfoxide and Temik sulfone and that these products should be considered when evaluating the fate of Temik residues in dairy animals.

Because Temik sulfoxide is unstable in the pure form, Temik was used in the present study. Based on the rapidity with which Temik was converted to its sulfoxide in rats (Andrawes *et al.*, 1967), a fate study of Temik would yield results comparable to those obtained should Temik sulfoxide be the administered compound. The rapid conversion of Temik to Temik sulfoxide in a lactating cow also was noted (Dorough and Ivie, 1968). Even in the urine, which contained over 90% of the dose given, Temik was not detected. However, Temik sulfoxide accounted for over 50% of the total Temik equivalents in urine collected only 3 hours after treatment. Thus, the 1 to 1 molar ratio of Temik and Temik sulfone administered to the cows in the current study was intended to represent, as closely as possible, actual residues that could be consumed by cows if fed crops treated with Temik for insect control.

Radioactive Temik was used in an attempt to obtain a complete picture of its metabolic fate in dairy cows. Admittedly, total detectable residues, and consequently the number of unknown metabolites, are probably increased over those observed had more selective analytical methods been employed. However, with selective methods, the scope of any metabolic fate or residue study is limited to a predetermined set of conditions. There is little likelihood that products of an unpredictable nature would be detected. This could result in toxicologically significant metabolites being overlooked in the environment and/or in products destined for human consumption. Radiotracer techniques do not assure the identity of all residues but they do assure that the presence of most metabolites is recognized. This way, it is possible for them to be evaluated as to general chemical nature, concentration, and detectability. De-

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Table I. Designations Used for Temik and Its Metabolites and Their Separation by TLC

Structure	Designation[a]	R_f Values[b] 1	2
$C^{14}H_3SC(CH_3)_2CH{=}NOC(O)NHCH_3$	Temik-C^{14}	0.67	0.94
$CH_3S(O)C(CH_3)_2CH{=}NOC(O)NHCH_3$	Temik sulfoxide	0.05	0.18
$C^{14}H_3S(O)_2C(CH_3)_2CH{=}NOC(O)NHCH_3$	Temik sulfone-C^{14}	0.21	0.64
$CH_3SC(CH_3)_2CH{=}NOH$	Temik oxime	0.78	0.95
$CH_3S(O)C(CH_3)_2CH{=}NOH$	Oxime sulfoxide	0.18	0.22
$CH_3S(O_2)C(CH_3)_2CH{=}NOH$	Oxime sulfone	0.56	0.78
$CH_3S(O)C(CH_3)_2C{\equiv}N$	Nitrile sulfoxide	0.31	0.70
$CH_3S(O)_2C(CH_3)_2C{\equiv}N$	Nitrile sulfone	0.62	0.93
Unknown	Unknown 1	0.05	0.11
Unknown	Unknown 2	0.10	0.24
Unknown	Unknown 3	0.11	0.34
Unknown	Unknown 3a	0.36	0.41
Unknown	Unknown 5	0.47	0.86

[a] Metabolite designations from Dorough and Ivie (1968). [b] Two dimensional TLC. First solvent system, 2:1 ether-hexane + 20% acetone and second system, 2:1 methylene chloride-acetonitrile.

pending upon these factors, judgment may be made as to the need for additional characterization.

EXPERIMENTAL

Animals. Holstein cows purchased from a commercial dairy were used in this study. One animal was used in a pilot study to determine the effect of the high Temik dose on the general health of the cow. The animal weighed 645 kg. and was in the latter stage of lactation. Three additional cows were used in the radioactive Temik feeding study. Each had calved 6 to 8 weeks previously and was in peak milk production. The cow fed the low level of Temik weighed 470 kg., that fed the medium level 500 kg., and the one fed the high level weighed 518 kg. at the beginning of the experiment. It was incidental that the weights of the cows increased with the dosage rates. In fact, the animals were placed in designated stalls prior to being weighed and by persons unfamiliar with the fate of the animals.

The animals were held in metabolism stalls and a 12-hour milking schedule was maintained. Milking was done by machine with separate apparatus used for each cow to minimize chances of cross contamination. The close proximity of the cows proved to be far superior to the single isolated animal situation used in earlier investigations. There was little sign of nervousness by the cows, and they responded to handling in a normal manner. More important, there was no decline in milk production owing to confinement as had often been the case with an isolated cow.

For the first 10 days of the study, during which the feces and urine were not collected, the cows were exercised daily. However, the cows were held in constant confinement during the next 14 days while being fed radioactive Temik and while the urine and feces were collected separately.

The urine was collected by attaching a flexible vinyl hose, 1 inch in diameter, to the vulva of the cow. First, the hose was fixed to a triangular pad constructed of vinyl tape in such a manner that the opening of the hose could be positioned directly over the vulva. Oils on the skin surrounding the vulva were removed by washing with ether, and then a layer of contact cement was applied. Contact cement was applied to the triangular pad also. When the cement on both surfaces was dry, the pad was attached to the vulva. To prevent the hose from falling beneath the animal when she was in a prone position, it was suspended from the ceiling with rubber tubing so that it was held away from the animal and about 12 inches from the floor at the lowest point. Collecting the urine by this method created no apparent problem for the cows and proved sufficiently stable so that only one hose had to be repaired during the 14-day test period.

To take samples of blood at frequent intervals without exciting the cows, an intervenous catheter was inserted into the jugular vein of each animal before treatment began. The catheter was equipped with a receptacle for a standard syringe with which the blood was withdrawn. As there was no pain associated with sampling the blood using this technique, it was unnecessary to restrain the animals.

At the morning and evening milkings each cow was given 12 pounds of grain. Water and alfalfa hay were provided *ad libitum*. The amount of feed consumed by each animal was recorded daily.

Treatment and Sampling. Temik-*S*-methyl-C^{14} and Temik sulfone-*S*-methyl-C^{14}, both having a specific activity of 5 mCi. per mmole, were obtained from the Union Carbide Chemicals Co. as were nonradioactive forms of these chemicals and metabolite standards (Table I). Equal molar quantities of Temik and Temik sulfone were dissolved in acetone and appropriate aliquots of the solution transferred to a gelatin capsule containing crushed grain. The capsules were administered to the cows with a balling gun twice daily at 12-hour intervals, one at the morning milking and one at the evening milking. For each treatment, feeding was begun and then, after several minutes, was interrupted briefly when the cow was given the capsule. The cows were milked immediately thereafter.

Dosage rates of Temik were calculated on the basis of an anticipated total feed intake of 50 pounds per cow per day. For example, the cow receiving 1.2 p.p.m. Temik equivalents in the diet was given 0.036 mmoles, 6.8 mg., of Temik and 0.036 mmoles, 7.9 mg., of Temik sulfone at each feeding.

The cow used in the pilot study was given nonradioactive Temik and Temik sulfone at a rate equivalent to 1.2 p.p.m. of Temik in the diet for a total of 10 days. Animals in the dosage series were fed nonradioactive insecticide for 10 days and then the radioactive products for an additional 14 days. In all cases, treatment was continuous throughout the indicated time periods.

Blood was taken daily from each cow while on the Temik treatment. Samples were withdrawn 6 hours after the morning milking and were immediately assayed for plasma and red blood cell (RBC) cholinesterase activity. Blood from animals fed radioactive Temik was also radioassayed to determine the total Temik equivalents present. Milk, urine, and feces were collected at 12-hour intervals, weighed, and then frozen until analyzed. Eighteen hours after the last radioactive Temik treatment, the cows were slaughtered and tissue samples removed for analysis.

Assay Procedure. For all radioactive measurements reported herein, a Packard Tri-Carb Model 3365 instrument was used. The scintillation mixture consisted of a 2 to 1 mixture of toluene and methyl cellosolve containing 5 grams of PPO per liter. All fluid samples and extracts were assayed directly by counting aliquots of 0.2 to 0.5 ml. Total radioactivity in the blood, tissues, feces, and substrate solids after extraction was determined by oxygen combustion in a Parr double-valved bomb. Approximately 1 gram of whole blood was placed in a small bag made from cellulose dialysis tubing (Kelly *et al.*, 1961) and dried overnight at 40° C. The dried

sample was combusted in oxygen at 25 atm. pressure, and the resulting carbon-14 dioxide was trapped in 20 ml. of a mixture of 2 to 1 methyl cellosolve and monoethanolamine (Jeffay and Alvarez, 1961). A 2-ml. aliquot of the trap solution was radioassayed by liquid scintillation counting.

For determination of blood cholinesterase levels, freshly drawn heparinized blood was centrifuged to separate the RBC's from the plasma. After the plasma was decanted, the RBC's were diluted to the original blood volume with distilled water. They were not washed prior to dilution. Cholinesterase activity in the plasma and RBC's was measured using a radioisotopic method (Reed *et al.*, 1966).

Extraction. Radioactive residues were extracted from the milk using the basic procedure of Timmerman *et al.* (1961). Briefly, the method called for the thorough mixing of whole milk, 50 ml., with acetonitrile to precipitate the milk solids, and then for the addition of chloroform so that an aqueous and an organic solvent layer was obtained. With the original method, the residues in the two liquid phases and in the milk solids would be quantitated at this point. In the present study, the aqueous phase was further concentrated to approximately 10 ml. and reextracted in a manner identical to the whole milk. This allowed more complete removal of the solids suspended in the aqueous portion of the milk and resulted in greater extraction of radioactive residues into the organic solvent fraction. For future reference, residues in the organic solvent fraction will be referred to as organo-extractables, those in the water layer as water-solubles, and radioactive residues remaining in the milk solids as unextractables.

Urine was extracted with chloroform, and the organo-extractable metabolites were characterized using the same techniques described for evaluating the nature of the organo-extractables from milk. Partial cleanup of the water-soluble metabolites from urine was accomplished on a Sephadex column. Sephadex LH-20, after being placed in distilled water for 3 hours and then in acetone for 10 minutes, was added to a chromatographic column, 2.5 cm. in diameter, until a column bed of 10 cm. was attained. The column was then washed with 50 ml. of acetone. Five milliliters of the chloroform-extracted urine was concentrated to approximately 0.2 ml. and transferred to the column. One hundred milliliters of acetone was passed through the column to elute any organo-extractable metabolites remaining in the urine after extraction with chloroform. This was followed by 100 ml. of a 6 to 1 mixture of acetone and methanol and finally by 100 ml. of a 1 to 1 mixture of acetone and methanol. The 6 to 1 solvent system served to remove certain interfering materials. Those radioactive materials eluted with the 1 to 1 solvent mixture were considered as the true-water-soluble metabolites.

The liver was the only tissue in which attempts were made to extract the radioactive products. Acetone, benzene, *n*-butanol, ethanol, methanol, water, and mixtures thereof were used in efforts to extract the residues.

Thin Layer Chromatography. Techniques used to separate and identify the radioactive organo-extractable metabolites were the same as reported earlier (Andrawes *et al.*, 1967; Dorough and Ivie, 1968). Metabolite designations are those used by Dorough and Ivie (1968). Their separation by thin layer chromatography is shown in Table I.

After elution from the column, the water-soluble metabolites from urine were resolved by thin-layer chromatography (TLC) using plates prepared from Silica Gel G slurried in a 0.1*M* boric acid solution. The chromatograms were developed in a 5 to 4 to 1 mixture of acetone, *n*-butanol, and 0.1*M* boric acid. After the radioactive areas on the plates were located by radioautography, they were extracted from the gel with methanol.

Table II. Treatment Rates and Feed Consumption for Dairy Cows Fed Temik for 24 Days[a]

	Average per Day Values/Cow No.			
	Pilot	1	2	3
Temik equivalents in feed, p.p.m.	1.2	0.12	0.6	1.2
Temik equivalents, mg./kg. (body wt.)	0.042	0.006	0.027	0.052
Feed consumption, lbs.	27	46	48	47
Milk production, lbs.	30	41	50	58

[a] The pilot cow was given nonradioactive Temik for 10 days while the other animals received the insecticide for a total of 24 days, 10 days on nonradioactive material and 14 days on radiolabeled products.

Analysis of Water-Soluble Metabolites. Attempts were made to cleave the aglycones from the urine water-soluble metabolites by acid and enzymatic hydrolysis. For acid hydrolysis, each metabolite(s) extracted from the silica gel was incubated in 2*N* HCl for 30 minutes at 95° C. Similarly, each metabolite(s) was incubated in various enzyme preparations at 37° C. for as long as 3 days. Enzymes (Sigma Chemical Co.) used in these studies and the pH of the incubation media were as follows: beta-glucuronidase Type H-1, pH 5.0; beta-glucuronidase Type 1, pH 6.9; beta-glucosidase, pH 5.3; sulfatase, pH 5.0; protease, pH 7.5; maltase, pH, 6.4; and alpha-amylase, pH 6.9.

Following incubation, the mixtures were extracted with chloroform and the percentage conversion of water-solubles into organo-extractables was determined. The radioactive components of the chloroform extract were separated by TLC and their chromatographic behavior was compared with Temik metabolite standards and with the original organo-extractable metabolites from the urine.

RESULTS

Effect on Animals. The pilot study indicated that feeding a dairy cow a diet containing 1.2 p.p.m. Temik equivalents would not cause any visible ill effects to the animal. Moreover, the blood cholinesterase levels were not reduced and the quantity of milk produced by the cow remained constant. Hay and grain consumption, 27 pounds per day, and milk production, 30 pounds per day, were well below that for the other animals used in this study (Table II). However, these values were considered normal for this particular cow since they were the same as the pretreatment figures. The fact that this cow was in the latter stage of lactation and was isolated from other animals during the feeding experiment could account for the low feed consumption and milk production. Urine and feces from the pilot cow were not quantitated.

Even though the pilot cow did not consume 50 pounds of feed per day, the animal was administered that amount of Temik and Temik sulfone which would be present in 50 pounds of feed containing 1.2 p.p.m. of Temik equivalents. Since this dose of insecticide was not harmful to the animal, the Temik feeding study involving three cows was initiated.

As observed in the pilot study, there were no apparent harmful effects to the cows resulting from Temik in the diet at 0.12 p.p.m., 0.6 p.p.m., or 1.2 p.p.m. Blood cholinesterase levels were the same during the time Temik was being fed as they were before treatment commenced. Milk production,

Table III. Elimination of Radioactivity by Cows Fed Temik-C^{14} Daily for 14 Days at Rates of 0.12, 0.06, and 1.2 P.P.M. in the Diet

Days Fed Insecticide	Per Cent of Total Dose for Each Treatment Level in[a]								
	Milk, p.p.m.			Urine, p.p.m.			Feces, p.p.m.		
	0.12	0.6	1.2	0.12	0.6	1.2	0.12	0.6	1.2
½	0.9	0.5	0.7	68.7	75.6	74.4	1.3	0.5	0.8
1	0.8	0.7	1.1	82.0	81.9	83.6	2.0	1.1	1.6
2	0.7	0.8	1.2	86.0	85.1	85.8	3.1	2.0	1.9
3	0.7	0.8	1.2	90.1	89.7	89.7	3.3	2.4	2.3
7	0.8	0.9	1.3	90.7	88.5	90.8	3.4	2.5	2.6
10	0.8	0.9	1.3	90.9	90.5	90.4	3.5	2.8	2.8
12	0.9	0.9	1.3	93.1	91.2	91.0	3.5	2.9	2.8
14	0.9	0.9	1.3	93.8	91.6	92.1	3.5	3.0	2.9

[a] Calculations based on total dose consumed and total radioactivity eliminated by each indicated time.

Table IV. Radioactive Components in Milk of Cows Fed Temik-C^{14} at Rates of 0.12, 0.6, and 1.2 P.P.M. for 14 Days

Metabolites	P.P.B. at Selected Days and 14-Day Average											
	Cow No. 1 (0.12 p.p.m.)				Cow No. 2 (0.6 p.p.m.)				Cow No. 3 (1.2 p.p.m.)			
	1	7	14	14-day av.	1	7	14	14-day av.	1	7	14	14-day av.
Organo-extractables[a]												
Temik sulfoxide	0.04	0.06	0.10	0.06	0.28	0.19	0.15	0.22	0.54	0.38	0.48	0.40
Temik sulfone	0.14	0.22	0.28	0.21	0.70	0.92	0.86	0.85	3.23	2.43	2.28	2.24
Oxime sulfoxide	0.04	0.01	0.09	0.05	0.34	0.16	0.46	0.27	1.87	0.61	1.14	1.13
Oxime sulfone	0.08	0.04	0.06	0.07	0.45	0.08	0.31	0.34	1.10	0.65	1.16	0.94
Nitrile sulfoxide	0.04	0.04	0.05	0.05	0.24	0.15	0.15	0.17	0.93	0.88	0.52	0.61
Nitrile sulfone	0.22	0.35	0.67	0.44	1.33	2.71	2.90	2.22	1.91	4.24	4.27	3.71
Unknown 1	0.01	0.01	0.02	0.01	0.03	0.05	0.05	0.04	0.14	0.08	0.11	0.08
Unknown 2	0.03	0.04	0.04	0.04	0.11	0.13	0.08	0.12	0.40	0.31	0.11	0.27
Unknown 3	0.04	0.09	0.15	0.08	0.52	0.36	0.44	0.29	1.40	0.83	0.50	0.74
Unknown 3a	0.06	0.07	0.11	0.08	0.23	0.18	0.14	0.22	1.05	1.08	1.14	0.96
Unknown 5	0.01	0.02	0.01	0.02	0.04	0.08	0.05	0.06	0.06	0.14	0.10	0.11
Water-solubles	0	0	0	0	0	0	0	0	0	0	0	0
Milk solids	0.20	0.24	0.3	0.28	0.94	0.83	0.90	0.91	2.52	1.94	1.80	2.06
Total	0.91	1.19	1.88	1.39	5.21	5.84	6.49	5.71	15.25	13.57	13.61	13.25

[a] Metabolites extracted from whole milk with acetonitrile and chloroform.

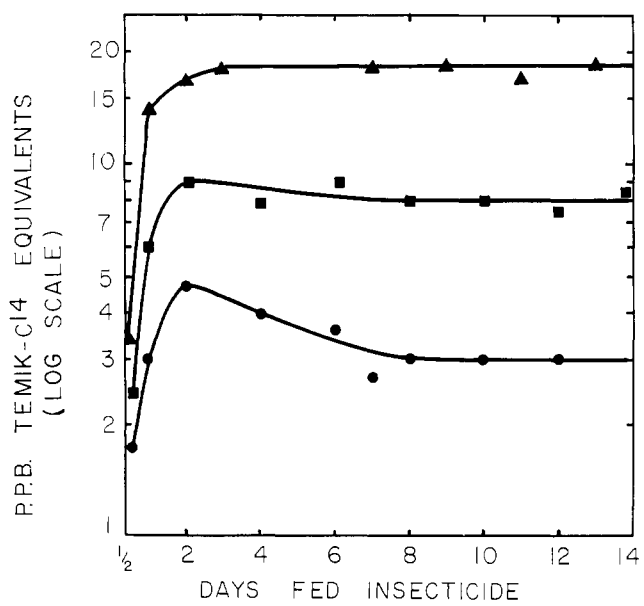

Figure 1. Temik-C^{14} equivalents in blood of cows while receiving Temik in the diet

Parts per million Temik in the feed; ● 0.12, ■ 0.6, ▲ 1.2

feed consumption, and quantity of excretory products remained stable throughout the experiment. Each animal consumed approximately 47 pounds of feed per day but varied slightly in the amount of milk produced (Table II). However, there was little day-to-day variation in the amount of milk produced by an individual animal.

Elimination of Total Temik Equivalents. The amount of the administered radioactive Temik eliminated from the cows in the milk, urine and feces is shown in Table III. Although all samples collected were analyzed, only those data sufficient to demonstrate the pattern of elimination are presented. The quantity of radioactivity eliminated by these three routes stabilized very rapidly. After 3 days, the concentration of Temik-C^{14} equivalents in the milk, urine, and feces was very close to the maximum detected at any time during the study. The fact that there was a consistent relationship between the amount of Temik consumed and the rate of elimination of residues from the body was apparent from the level of radioactivity detected in the blood (Figure 1). After 2 days on the Temik diet, residues of Temik-C^{14} equivalents in the blood of the cow fed 0.12 p.p.m. were maintained at approximately 3 p.p.b. The blood of animals fed Temik at 0.6 and 1.2 p.p.m. contained approximately 8 and 18 p.p.b., respectively, after the second day of feeding.

Table V. Radioactive Components in Urine of Cows Fed Temik-C^{14} at Rates of 0.12, 0.6, and 1.2 P.P.M. for 14 Days

Metabolites	P.P.B. at Selected Days and 14-Day Average								
	Cow No. 1 (0.12 p.p.m.)			Cow No. 2 (0.6 p.p.m.)			Cow No. 3 (1.2 p.p.m.)		
	1	14	14-day av.	1	14	14-day av.	1	14	14-day av.
Organo-extractables[a]									
Temik sulfoxide	4.3	4.1	4.4	27.2	24.0	25.6	47.0	48.3	43.6
Temik sulfone	8.4	6.8	7.3	39.5	28.0	35.9	63.0	72.5	66.5
Oxime sulfoxide	6.5	7.1	7.7	24.6	29.5	34.7	60.9	115.2	96.4
Oxime sulfone	4.6	4.7	4.9	31.1	25.6	32.3	38.4	51.8	43.6
Nitrile sulfoxide	0.5	0.9	1.0	2.6	2.5	3.6	9.6	10.3	8.0
Nitrile sulfone	0.5	2.3	1.5	2.6	6.0	6.7	4.2	16.1	10.3
Unknown 1	0.2	0.1	0.1	0.6	0.5	0.6	1.0	1.1	1.1
Unknown 2	0.5	0.7	0.6	3.2	2.0	2.4	1.2	12.6	6.8
Unknown 3	0.6	1.4	1.1	3.8	10.0	7.9	2.1	11.5	8.0
Unknown 3a	1.2	1.7	1.2	1.9	2.5	3.6	2.1	19.5	16.0
Water-solubles	125.8	91.1	96.0	551.5	370.7	465.2	835.9	792.5	847.2
Total	153.1	120.9	125.8	688.6	501.3	609.5	1065.4	1151.4	1147.5

[a] Metabolites extracted from urine with chloroform.

Most of the consumed Temik-C^{14} was eliminated from the cows in the urine (Table III). Regardless of the level of treatment, about 90% of the daily dose could be accounted for in urine collected the following day. There was a slight, but continuous, increase in the percentage of the dose eliminated in the urine during the feeding of radioactive Temik. This may have resulted from an increase in efficiency of elimination of the daily doses by the animals or from the release of products stored from the earlier feedings. The same type of increase was noted in the feces. Whereas from 1 to 2% of the dose was voided in the feces after the first day, 3 to 3.5% of the cumulated C^{14}-treatments was eliminated in the feces by the 14th day. By adding the percentage of the dose eliminated in the milk, approximately 1.0%, to the corresponding values for urine and feces, it was concluded that dairy animals on a continuous diet containing Temik would consistently eliminate 90% or more of the insecticide consumed daily.

Milk. Parts per billion of Temik-C^{14} equivalents in the milk were directly related to the concentration of Temik in the diet, Table IV. The level of C^{14}-residues in milk from the cow fed 0.12 p.p.m. of Temik ranged from 0.9 to 1.9 p.p.b. and averaged 1.4 p.p.b. For the 0.6 p.p.m. feeding level, the residues in the milk ranged from 5.0 to 6.5 p.p.b. and at the 1.2 p.p.m. level, the residues ranged from 12.1 to 15.3 p.p.b. The average concentrations of Temik-C^{14} equivalents in milk resulting from the latter two feeding levels were 5.7 and 13.3 p.p.b., respectively. The ranges cited above do not include values obtained from samples collected within 12 hours of the first treatment. Radiolabeled residues had not reached the point of stabilization at that time and did not represent a true continuous feeding situation. The average values include data from 28 separate milk samples for each feeding level, two samples each day for 14 days.

The nature of the radioactive residues in the milk is shown in Table IV. Again, analyses were performed on each milk sample collected throughout the feeding study, but only selected data are presented because the results were almost identical for all samples within a given treatment. As demonstrated in Table IV, the relative concentration of metabolites in the milk remained fairly constant during the 14-day feeding period. These results indicated that the metabolism of Temik was not altered by continuous feeding of the insecticide or by increasing its concentration in the diet from 0.12 to 1.2 p.p.m.

Oximes and nitriles made up approximately 50% of the radiolabeled metabolites. Nitrile sulfone was the major metabolite, accounting for approximately 60% of the known hydrolytic products and 30% of all radioactive residues in the milk.

Temik sulfone was the principal carbamate in the milk, constituting from 15 to 19% of the radioactive products present. Temik sulfoxide was present at about one-fourth this level. Their combined concentrations were 0.3, 1.0, and 2.7 p.p.b. in milk from cows fed Temik at the three dosage levels. The parent compound, Temik, was not detected in any of the milk samples.

Collectively, the organo-extractable metabolites listed as unknowns in Table IV composed 15% of the total residues. However, the maximum concentration of these unknown metabolites, even in milk of the cow fed the high level of Temik, was only 2.2 p.p.b.

After concentrating the aqueous fraction of the milk extract and thoroughly re-extracting it with acetonitrile and chloroform, there were no detectable water-soluble metabolites present in the milk (Table IV). Without this additional extraction, however, from 10 to 15% of the radioactivity in the milk remained in the water phase. Independent analysis of these water-soluble products revealed that approximately 65% were bound with the milk solids, 15% as Unknown 3, 10% as Temik sulfoxide, 5% as Temik sulfone, and 5% as Unknown 3a.

Radioactivity remaining with the milk solids after extraction accounted for 15 to 20% of the total labeled-residues in the milk. Although sizable when considered in relation to their percentage of the total residues, their absolute concentration was very low. These values were 0.3, 0.9, and 2.1 p.p.b. for the three treatment rates, respectively (Table IV). No attempt was made to characterize the radioactive metabolites of Temik located in the solid fraction of the milk.

Urine. Radioactive metabolites of Temik in the urine increased proportionally with the increased levels of Temik in the diet of the cows (Table V). As was the case in the milk, however, the relative concentrations of the metabolites were strikingly similar regardless of the levels of Temik fed.

Only about 25% of the radiolabeled products in the urine

Table VI. Thin Layer Separation[a] of Radioactive Water-Soluble Metabolites in Urine of Cows Fed 1.2 P.P.M. Temik-C^{14} for 14 Days

Radioactive Band	R_f	Per Cent of Water Solubles/Days 3	7	10	14
1	0	5.1	5.8	3.1	5.0
2	0.1	42.2	40.4	46.7	38.9
3	0.17	42.8	46.4	42.0	40.9
4	0.39	9.9	7.4	8.2	15.2

[a] TLC plates prepared with Silica Gel G in 0.1 *M* boric acid. Solvent system consisted of a 5:4:1 mixture of acetone, *n*-butanol, and 0.1 *M* boric acid.

was extractable with chloroform. Of these, approximately 40% was identified as Temik sulfoxide and Temik sulfone, 50% as oximes and nitriles, and 10% was unknown materials. At the low feeding level, the average concentrations of these products were 11, 14, and 3 p.p.m., respectively. Urine from cows fed 0.6 and 1.2 p.p.m. contained residues corresponding to the increased dosage rates. With the exception of Unknown 5, found only in the milk, the organo-extractable metabolites in the milk and urine were the same.

Seventy-five per cent of the radioactivity in the urine remained in the aqueous phase after extraction with chloroform (Table V) and was resolved by TLC into four distinct radioactive bands (Table VI). When unextracted urine was concentrated and applied to the chromatograms, two additional bands were observed, one with a R_f value of 0.60, band 5, and the other a R_f of 0.76, band 6. Examination of these bands individually on TLC, using the solvent systems for organo-extractables, showed that the R_f 0.6 material cochromatographed with Temik sulfoxide and that the R_f 0.76 band was a mixture of other organo-extractable metabolites. In this same solvent system, the radioactive materials of bands 1 through 4 (Table VI) stayed at the origin.

TLC analysis of chloroform-extracted urine disclosed that small amounts of bands 5 and 6 were still present, demonstrating that the partitioning characteristics of the organo-soluble metabolites make it virtually impossible to attain complete extraction. Although their combined concentration was only 1 to 3% of the radioactivity in the extracted urine, it was sufficient to interfere with subsequent analysis of the water-soluble metabolites. This problem was solved by removing the organo-extractable radioactivity from the urine by Sephadex column chromatography. That material eluted from the column with a 1 to 1 mixture of acetone and methanol contained only the four radioactive bands shown in Table VI. The organo-extractable materials were eluted beforehand with acetone. In addition to allowing complete separation of the two classes of metabolites, the Sephadex column removed much of the interfering material from the radioactivity and transferred the water-soluble metabolites to an organic solvent. The latter could be more readily reduced in volume for application to TLC and gave improved separation of metabolites.

Water-Soluble Metabolites. Cleavage of water-soluble metabolites of Temik from urine by enzymatic means was almost totally unsuccessful. The maximum conversion of water-soluble metabolites to organo-extractable materials came when the incubation mixture consisted of beta-glucuronidase Type H-1 in pH 5 buffer. After 3 days at 37° C, 7% conversion had taken place. Increasing the amount of enzyme and/or the period of incubation failed to increase the quantity of organo-extractable materials. Two-dimensional TLC chromatography (Dorough and Ivie, 1968) of the aglycones showed the presence of three products, one chromatographing with Unknown 3 of the original organo extractables, one chromatographing with Unknown 3a, and the other chromatographing with oxime sulfone. Exact quantitation and identification were not possible because of the small quantity of radioactivity.

Acid hydrolysis of the water-soluble metabolites yielded much higher amounts of organo-solubles than did the enzymatic method. For these studies, radioactive bands 1 through 4 (Table VI) were incubated separately in 2*N* HCl at 95°C for 30 minutes. Each of the bands yielded several organo-extractable products after being resolved by TLC. However, there was a single major product produced in every case. With band 1, 72% of the water-solubles was cleaved by the acid, and 95% of these were in the form of an unknown which was designated as Unknown A. Band 2 was hydrolyzed 80% by the acid and 96% of the aglycones was as a material designated Unknown B. Acid hydrolysis of band 3 gave only 45% cleavage of the water-soluble materials while 73% of band 4 was cleaved. Both band 3 and 4 aglycones were in the form of Unknown B in excess of 95%.

Unknowns A and B were products which did not cochromatograph with any of the metabolite standards of Temik. Thus, the watersolubles in the urine were not conjugates of the free Temik metabolites or the free metabolites, if formed by acid hydrolysis, were unstable in the incubation medium. Incubating the Temik metabolite standards in acid and then examining the products extracted with chloroform showed that Temik and the standards were highly unstable under these conditions. This made it impossible to identify the aglycones as they existed as part of the water-soluble metabolites.

Since the enzymatic cleavage of the water-soluble metabolites failed and the acid cleavage resulted in the destruction of the aglycones, only indirect evidence is available concerning the identity of the Temik-contributing portion of the conjugate metabolites. When Unknown A and Unknown B, formed by acid hydrolysis of the conjugates, were compared with the unknowns produced from the Temik standards upon acid hydrolysis, certain products were identical chromatographically. The major acid degradation product of oxime sulfone cochromatographed with Unknown B. That oxime sulfone was the only standard yielding Unknown B suggested that it could be the major component of the water-soluble metabolites of Temik in the urine. In fact, over 95% of the radioactivity in bands 2 through 4 (Table VI) was degraded to the same product by acid hydrolysis as was oxime sulfone. Only band 1, which remained at the origin of the TLC, was degraded to a large extent to Unknown A. This unknown was not the principal degradation product of any of the Temik standards when placed in the acid hydrolysis conditions. However, it did cochromatograph with Unknown 3 of the organo-extractable metabolites. It is possible, therefore, that approximately 5% of the urine water-solubles was conjugates of an unknown aglycone and that which remained was conjugates of oxime sulfone. While positive identification of the conjugate metabolites of Temik must await further evaluation, these data offer strong evidence that Temik, Temik sulfoxide, and Temik sulfone, which are of obvious toxicological importance, do not directly contribute to their formation.

Tissues. Of 27 different tissue samples analyzed for total Temik-C^{14} equivalents, detectable residues were observed in 22 tissues from the cow fed 1.2 p.p.m. Temik in the diet, in

20 tissues from the cow fed 0.6 p.p.m. Temik, and 1 sample taken from the cow fed 0.12 p.p.m. Temik (Table VII). Even at the high feeding level, residues were absent in muscle tissue, fat, and bone. With the exception of those in the liver, all of the residues in the tissues from the cow fed 0.6 p.p.m. Temik were considered as trace quantities since they were present at levels only slightly above the limit of sensitivity of the analytical method. Generally, the same was true for tissues from the cow fed the high level of Temik. Only the liver and lungs contained residues in excess of fourfold the 4 p.p.b. limit of sensitivity. In this animal, the lungs contained 35 p.p.b. Temik-C^{14} equivalents and the liver 164 p.p.b. Temik-C^{14} equivalents.

The liver from the animal fed the highest concentration of Temik was the only tissue in which the radioactive content was sufficient to warrant extraction and characterization of the residues. However, attempts to extract the radioactive residues failed, and nothing of their nature was determined. The fact that they resisted extraction so successfully might suggest that the residues in the liver were not Temik-like at all but were naturally occurring products containing a mere fragment of the Temik molecule. It is unlikely that products other than these would remain with the solid liver residue rather than being in the organic solvent or water phase after extraction with water, acetone, methanol, *n*-butanol, or hot ethanol as was found to be the case.

Analytical Considerations. The present study showed that the parts per million level of total residues in the milk of cows fed Temik were approximately $^1/_{100}$ that level in the diet (Table IV). This relationship held true for feeding levels varying from 0.12 to 1.2 p.p.m., and in animals where the average milk production varied from 41 to 58 pounds per day. Because of this, one should be able to predict with a high degree of accuracy the concentration of residues in milk when the level of Temik in the diet is known. However, the actual quantitation of Temik equivalents in milk may be difficult.

Since the residues in milk of cows fed Temik-contaminated feed were so low, it is unlikely that they would be detected by conventional analytical methods. Even on a total Temik-equivalent basis, a method would have to be sensitive below the 0.01-p.p.m. level to detect residues in milk of animals fed 1 p.p.m. Temik in the diet. To detect only the known carbamate materials, Temik sulfoxide and Temik sulfone, the sensitivity of the method would have to be greater than 0.002 p.p.m. Lower feeding levels of Temik would obviously demand even greater sensitivity if residues were to be quantitated.

For monitoring purposes and for certain investigational uses, it would be possible to use the urine as an indicator of residue levels in the milk. The p.p.m. Temik equivalents in urine (Table V) were approximately 100 times greater than those in the milk, and the same as the p.p.m. Temik fed in the diet. It would be possible to detect combined Temik sulfoxide and Temik sulfone in urine if cows were fed Temik in the diet at concentrations as low as 0.12 p.p.m. This would require a sensitivity of only 0.01 p.p.m. If the two compounds were detected, it would indicate that their combined concentration in the milk (Table IV) was about one-fiftieth that observed in the urine. If total residues were detected, the indicated residues in the milk would be $^1/_{100}$ that in the urine.

Comparison of Single-Dose and Continuous-Feeding Studies. Generally, there was good agreement between results reported by Dorough and Ivie (1968) and those obtained in the current tests. In the earlier study, Temik-S^{35} was given as a single oral dose at a rate equivalent to approximately 3.5 p.p.m. in the diet. During the first 24 hours after treatment, 83% of the dose was eliminated in the urine and 1% in the milk. These values are very close to that amount eliminated 24 hours after the first Temik–Temik sulfone doses (Table III). The feces of the Temik-S^{35} treated cow contained a lower percentage of the dose after 24 hours than did the Temik–Temik sulfone treated animals, 0.6% as compared with about 2%. However, the total eliminated by this route after the single treatment, 2.9% of the dose, was almost identical to the average daily values observed in the continuous feeding study.

Table VII. Radiolabeled Residues in Tissues of Cows Fed Temik-C^{14} for 14 Days[a]

Tissues[b]	P.P.B. Temik Equivalents at Indicated Feeding Level[c]		
	Cow No. 1 (0.12 p.p.m.)	Cow No. 2 (0.6 p.p.m.)	Cow No. 3 (1.2 p.p.m.)
Liver	29	123	164
Lungs	–	7	35
Kidney	–	6	16
Bile	–	9	16
Adrenal glands	–	6	12
Abomasum	–	4	11
Omasum	–	4	11
Large intestine	–	5	10
Ovaries	–	6	10
Rumen	–	5	10
Udder	–	6	10
Pancreas	–	5	9
Spinal cord	–	–	9
Gall bladder	–	4	8
Heart	–	6	8
Reticulum	–	6	8
Spleen	–	5	8
Skin	–	4	7
Small intestine	–	5	7
Brain	–	4	6
Neck muscle	–	–	6
Tongue	–	4	6

[a] Animals slaughtered 18 hours after last treatment. [b] Residues were not detected (below 4 p.p.b.) in the following tissues: foreleg muscle, hindleg muscle, omental fat, subcutaneous fat, and rib bone. [c] – indicates residues below 4 p.p.b.

The similarities between the results of the single- and continuous-Temik feeding studies were maintained to a large degree when the residues in milk were considered in detail. The average concentration of Temik equivalents in milk collected during the first 24 hours after the Temik-S^{35} treatment was 39 p.p.b., three times the 14-day average value in milk from the cow treated with Temik plus Temik sulfone at 1.2 p.p.m. The threefold increase was coincident with the higher treatment rate, 3.5 p.p.m. Temik in the diet, used in the single treatment study.

There were two differences noted when the chemical nature of residues in the milk from the single- and continuous-Temik feeding studies were compared. First, the 24-hour milk from the Temik-S^{35} study had 67% of the radioactivity in the organo-extractables, 23% in the aqueous milk phase, and 10% in the milk solids. Corresponding values for the Temik–Temik sulfone feedings were approximately 80, 0, and 20%. These differences may be explained by incomplete extraction and separation of the milk phases in the initial study. As pointed out earlier, improved extraction techniques were utilized in this experiment.

The second difference in the chemical nature of residues in milk from the two studies was the presence of metabolite Unknown 3a in the latter study (Table IV). This material was not detected in the Temik-S^{35} test but was evident in milk

from all three animals treated with Temik–Temik sulfone-C^{14} for 14 days. It also was detected in the urine of these cows. The fact that Unknown 3a was detected in samples of milk and urine collected within 1 day after treatment began shows that the metabolite did not result from continuous treatment. Therefore, the metabolite must be an initial metabolic product of Temik and/or Temik sulfone. Recent studies in our laboratory on the metabolism of Temik in chickens showed that metabolite Unknown 3a is present in the feces of birds treated with Temik-S^{35} and Temik-*S*-methyl-C^{14}. These data indicate that Unknown 3a was present in milk and urine of the cow treated with a single dose of Temik-S^{35}. Possibly, its presence was not detected because of the frequent intervals (3, 6, 12, and 24 hours after treatment) in which the samples were collected. Sampling in this manner could prevent the accumulation of a relatively slowly-formed metabolite to a detectable level.

With only minor exception, then, it is apparent that the continuous exposure of dairy animals to Temik in the diet does not significantly alter its fate as compared to a single exposure. Therefore, long-term feeding studies to determine the relationship of levels in the diet to residues in animal products when dealing with rapidly metabolized and rapidly excreted compounds such as Temik are not required. Also, it is evident that a single-dose study, usually designed to determine the general metabolic fate of a compound, can be very useful in estimating the concentration and nature of residues which might occur in consumable products of animals receiving insecticides of this type in the diet.

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