The Metabolism of Sevin in Dairy Cows

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Milk from cows on a diet containing Sevin was shown to be free from residues of Sevin, 1-naphthol (the hydrolysis product of Sevin), and detoxified conjugates of 1-naphthol. Part of the ingested Sevin appeared in the urine as 1-naphthol or Sevin or both. Another portion of the Sevin was found unchanged in the feces.

SEVIN INSECTICIDE (1-naphthyl *N*-methylcarbamate) is an effective agent for the control of a wide variety of insects, including many affecting forage crops. A study was initiated to determine if residues of Sevin remaining on these crops appear in milk of cows consuming the forage.

Previous work of this nature was done by Cornell University and the U. S. Department of Agriculture investigators (*2*). It was found that Sevin does not appear in milk from exposed cows and that it has no effect on the flavor and odor of the milk (*2*). However, the methods of analysis were incapable of establishing the presence or absence of metabolites of Sevin. This report describes work in two feeding experiments which were designed to investigate the metabolism of Sevin.

Analytical Methods

To determine the metabolic fate of ingested Sevin, methods of analysis were needed which would be capable of measuring accurately small quantities of residues in milk and excreta from exposed cows. Therefore, procedures were developed for Sevin, 1-naphthol (the hydrolysis product of Sevin), and conjugated detoxification products of 1-naphthol in milk, urine, and feces. These conjugates have been identified in the urine of exposed guinea pigs (*3*).

Analysis of Milk. Cream from portions of milk equilibrated and separated at 0° and 35° C. was analyzed for 1-naphthol and Sevin, respectively. For 1-naphthol, the distribution coefficient was 0.57, and for Sevin it was 0.5. The coefficients were determined by successive extraction of fortified skimmed milk with cream followed by analysis of the cream for the residues.

The aqueous portion of the milk from the 1-naphthol analysis was used for the determination of conjugates of 1-naphthol.

Determination of 1-Naphthol. Collect an 800-gram sample of fresh, whole milk in a separatory funnel and reduce the temperature immediately to 1° to 2° C. Equilibrate the sample by intermittent shaking for 5 minutes. Store the funnel at 2° C. for 24 hours to allow complete separation of the phases.

Extract the cream with 150 to 200 ml. of methylene chloride in a laboratory blender. Transfer the mixture to a separatory funnel. After complete separation of phases, reserve the methylene chloride layer and re-extract the cream phase with additional, fresh methylene chloride. Repeat the procedure once more, using enough methylene chloride to bring the volume of the combined extracts to 500 ml.

After filtering the combined extracts, remove the 1-naphthol by two extractions with a total of 25 ml. of 0.5*N* sodium hydroxide. Centrifuge the aqueous extracts and acidify the decanted upper layer with 3 ml. of concentrated hydrochloric acid. Equilibrate the acidified extract twice at 20° C. with a total of 35 ml. of methylene chloride. After filtration, remove one half of the solvent at 5° to 15° C. at reduced pressure. Pass the remaining solution through 35 mm. of 60- to 100-mesh Florisil containing 2.8% water. Elute the remaining 1-naphthol from the column with an additional 50 ml. of water-saturated methylene chloride. Combine the eluates in a 250-ml. flask and add 1 drop of diethylene glycol. After evaporation to dryness at 5° to 15° C. at reduced pressure, add 1.0 ml. of 0.1% sodium hydroxide in methanol to the flask. Add 9.0 ml. of glacial acetic acid. Add 1.0 ml. of a freshly prepared 0.1% solution of *p*-nitrobenzenediazonium fluoborate in acetic acid while swirling the solution. Refer the peak absorbance at 475 mμ in a 1.0-cm. cell, after correction for reagent blank, to a calibration curve prepared in the same manner except to omit the cream. The sensitivity of the method is 0.005 p.p.m. of 1-naphthol (Table II).

Determination of Sevin. Collect an 800-gram sample of fresh, whole milk in a separatory funnel and adjust the temperature to 35° C. After equilibration of the mixture by intermittent shaking for 5 minutes, mechanically separate the cream from the whole milk.

Extract the cream in the same manner as in the 1-naphthol determination. After filtration, evaporate the methylene chloride solution to butterfat in a Kuderna-Danish evaporator in a steam bath. Dissolve the butterfat layer in 150 ml. of petroleum ether and extract three times with acetonitrile at 10° to 15° C. After evaporation of the acetonitrile, take up the residues with 15 ml. of water-saturated methylene chloride and pass through Florisil, as in the 1-naphthol procedure. Elute the column with sufficient methylene chloride to bring the total volume to 100 ml. after which evaporate the solvent in the presence of one drop of diethylene glycol. Dissolve the residues in 2 ml. of 0.1*N* sodium hydroxide in methanol and allow to stand for 5 minutes at room temperature. Add 18 ml. of glacial acetic acid. With swirling, add 1 ml. of a freshly prepared 0.1% solution of *p*-nitrobenzenediazonium fluoborate from a pipet. Determine the absorbance under the same conditions used in 1-naphthol determination. The sensitivity of the method is 0.016 p.p.m. (Table II).

Determination of Conjugates of 1-Naphthol. Add 20 grams of skimmed milk to a 250-ml. flask containing 40 ml. of distilled water and 10 ml. of concentrated hydrochloric acid. Reflux the mixture at 103° C. under a West condenser for two minutes and cool to room temperature. After filtration, extract the solution with 25 ml. of methylene chloride at 10° C. Using up to 20 grams of anhydrous sodium sulfate and centrifugation, obtain a clear layer of methylene chloride. Equilibrate the methylene chloride layer with sodium hydroxide solution to remove the hydrolyzed conjugate residues. The remainder of the analysis is analogous to the 1-naphthol determination discussed earlier.

This method was based on data ob-

Table I. Sensitivity[a] of Method for 1-Naphthol Conjugates in Fortified Skimmed Milk

1-Naphthol Added, P.P.M.	Absorbance vs. Reagent Blank
0	0.008
	0.014
	0.004
0.13	0.031
0.27	0.055
0.50	0.122
0.54	0.131

[a] Sensitivity, = <0.13 p.p.m.

Table II. Recovery of Residues from Fortified Whole Milk[a]

P.P.M. Added	Absorbance vs. Reagent	P.P.M. Found	Recovery, %
	SEVIN		
0.000	0.024	0.004	...
0.021	0.078	0.016	76
0.032	0.100	0.025	78
0.043	0.160	0.044	102
0.053	0.179	0.051	96
	1-NAPHTHOL		
0.000	0.001	0	...
0.012	0.073	0.011	92
0.025	0.158	0.019	76
0.046	0.409	0.042	91

1-NAPHTHYL SULFATE (AS 1-NAPHTHOL)

P.P.M. Added	Absorbance vs. Control[b]
0	
0.08	0.031
0.13	0.047
0.21	0.102
0.26	0.118

[a] Residues added in aqueous solution to whole milk prior to equilibration and separation of cream.

[b] Obtained using fortified control milk. Plotted results were used as a calibration curve in the determination of residues in skimmed milk from exposed cows.

Table III. Analysis of Urine from Exposed Cow

Sample Designation	Days of Collection Relative to First Day of Exposure	Free 1-Naphthol and/or Sevin[a]	1-Naphthyl Sulfate, as 1-Naphthol, P.P.M.
Control	−1	0	0
Exposed	+3	1.1	66
	+3	1.3	65
	+3	1.2	66
Exposed	+10	1.1	65
Control	+17	0	8[b]

[a] Residues extracted from urine and saponified. Free and derived 1-naphthol determined as in method for Sevin in milk.

[b] This value obtained from absorbance due to red phenolic interference. Typical naphthol blue color not present.

Table IV. Rate of Elimination of Residues with Feces

Hours after Exposure	Weight of Sample	Indication of Residues
13	776	V. sl. positive
16.5	847	Strongly positive
19	928	Intense (18 p.p.m. Sevin; 1.3 p.p.m. 1-naphthol; <2 p.p.m. conjugates
21.7	821	Positive
36.5	2676	V. sl. positive

tained from the analysis of urine from cows exposed to Sevin. Such urine was found to contain water-soluble, acid-hydrolyzable derivatives of 1-naphthol, presumed at the time to be the sulfuric or the glucuronic acid conjugates of 1-naphthol or both. Later, using an enzymatic method, the derivative was established as 1-naphthyl sulfate (*7*).

The conditions required for quantitative acid hydrolysis of the conjugates were established for urine (Figure 1), and except for hydrolysis time and sample size, the same conditions were used for the milk samples.

The sensitivity of the method was shown to be approximately 0.1 p.p.m. (Table I). These results were obtained by adding 1-naphthol in aqueous dilution to the skimmed milk prior to acidification and reflux.

Methods of Analysis for Urine. Free 1-naphthol and Sevin were determined by extraction immediately and in the cold from an acidified solution of urine. Point (*a*) in Figure 1 shows that practically no hydrolysis of conjugates occurs under these conditions. The remainder of the methods are similar to those used in milk analysis.

Conjugates, as stated above, were determined by acid hydrolysis at 103° C.

Methods of Analysis for Feces. The analysis of feces for residues is complicated by the fact that 1-naphthol reacts with component(s) in feces in the presence of air to form a material which does not respond to the colorimetric test for 1-naphthol. The rate of disappearance of 1-naphthol in feces was 17 times more rapid in the presence of air than it was under a blanket of nitrogen.

Again, the methods are similar to those used in the analysis of milk. However, in the initial extraction, 10 grams of feces which has been protected from air are mixed with 40 grams of sodium sulfate and acidified prior to extraction with methylene chloride in a laboratory blender.

Feeding Experiments

Two feeding experiments were carried out. The major one, done at Cornell, supplied nearly all of the information. A supplementary experiment was performed locally (W. Va.) to confirm some data obtained from the analysis of spot feces samples collected in the Cornell experiment.

In the Cornell experiment, four Holstein milk cows confined to their stalls were fed 450 p.p.m. technical Sevin (98.3% active) daily for 14 days. The concentration of Sevin, which was based on intake of dry roughage, was determined daily and administered in equal amounts in 1 pound of grain at the morning and evening feedings. The animals received no additional food until the fortified grain was completely consumed. Two other Holstein cows, stalled adjacent to the exposed animals, served as controls throughout the test period. All animals received the same diet of grain, hay, and silage for the full 22 consecutive days of the experiment.

Sample Collection. The milk from each animal was collected by milking machine each evening and stored at 40° F. until morning. The evening sample was composited with an equal quantity of milk taken from the next morning's milking. The mixed sample was then processed for analysis of Sevin and 1-naphthol. This procedure was begun 3 days before the first day of exposure and continued 4 days after the last day of exposure.

Control urine from one of the exposed cows was collected on a spot basis prior to the first exposure. Twice during the test, once on the third and again on the 10th day, urine was collected from the same animal on a semi-quantitative basis. On the third day, the total urine volume was composited and sampled at various times throughout the period. On the 10th day of exposure, consecutive urinations were collected and frozen immediately. The procedure was repeated 4 days after the last exposure.

Feces samples were collected on a spot basis from the same animal before and during Sevin exposure.

Sample Preparation. Portions of milk for Sevin and 1-naphthol analysis were processed each day and the samples stored in polyethylene bottles and frozen.

For Sevin analysis, an 800-gram portion of milk was mechanically separated at 35° C. after equilibrating the milk at the same temperature. The separated cream was stored in polyethylene bottles in a freezer prior to analysis. Fortification of milk was done by adding Sevin in aqueous dilution to the milk prior to equilibration and separation. The resulting cream was stored and analyzed in the same manner as the samples.

In the preparation of samples for 1-naphthol analysis, 800-gram portions of composited milk were equilibrated at 1° to 2° C. and frozen immediately in polyethylene bottles. Prior to analysis, the samples were thawed, trans-

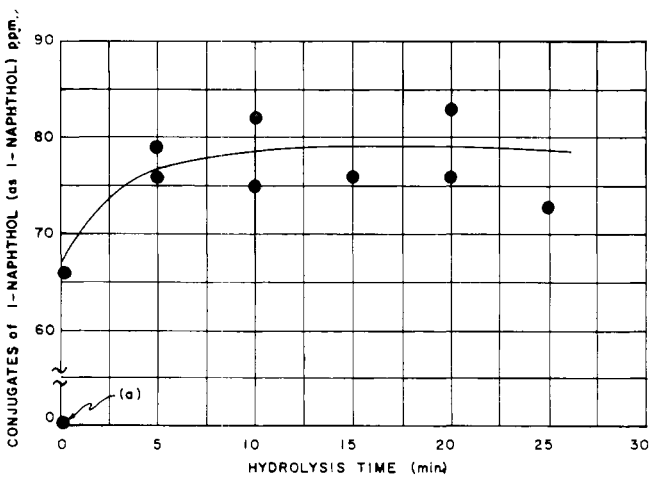

Figure 1. Determination of hydrolysis time of 1-naphthol conjugates in cow urine at 130° C.

[(a) hydrolysis temp = 20° C.]

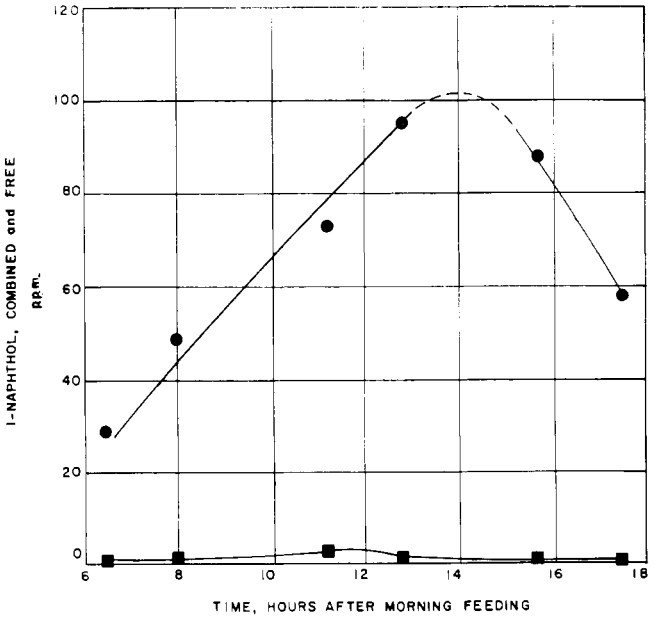

Figure 2. Analysis of urine from exposed cow on 10th day of test

(●—1-naphthol sulfate; ■—free 1-naphthol and/or Sevin)
Residue extracted from urine and saponified. Combined free and derived 1-naphthol determined as in method for Sevin in milk

ferred to separatory funnels, and equilibrated again at 1° to 2° C. They were then allowed to stand at the same temperature for 24 hours for complete separation of cream by gravity. Fortified samples were handled in exactly the same manner, except 1-naphthol in aqueous solution was added to the milk prior to the initial equilibration at 1° to 2° C.

The skimmed milk from the 1-naphthol samples was used for the determination of conjugates of 1-naphthol. The conjugates are water soluble and, if present, would occur in the aqueous phase of the milk.

Urine and feces samples required no processing when collected.

Results

Results from analyses of milk and excreta collected in the experiments showed that the milk was free from the residues which appeared in the excreta.

Analysis of Milk. No detectable amount of residues were encountered before, during, or after the exposed period. The sensitivities, determined from the fortified samples which were prepared daily during the experiment, were: Sevin, 0.016 p.p.m.; 1-naphthol, 0.005 p.p.m.; and conjugates (as 1-naphthol), 0.1 p.p.m. Recovery of residues was satisfactory. Some typical results are shown in Table II.

The failure of the cow to pass 1-naphthol or its conjugates into the milk was further demonstrated. A Holstein cow was fed daily doses of 1-naphthol at the rate of 12 p.p.m. on body weight. Milk collected during the 3-day feeding period contained no residues of 1-naphthol or conjugates. The urine during this period, however, contained approximately 10 times more conjugates than urine from the Sevin-exposed cows.

Analysis of Urine. To determine the route of exit of Sevin or its degradation products or both, the excreta of exposed cows were examined for Sevin, 1-naphthol, and 1-naphthol conjugates. A sample of control urine was collected from one of the exposed cows during the control period. This sample was used as a control for subsequent analyses performed on the urine. Table III shows that on the 3rd and 10th days of dosage, the urine contained about 65 p.p.m. 1-naphthol conjugates and a trace of free 1-naphthol or Sevin or both. Four days after the last exposure to Sevin the urine was again free of residues.

Hourly changes in conjugate content of the urine were measured from samples collected at each urination on the 10th day of feeding and compared with samples collected in a similar fashion 4 days after the last feeding. Figure 2 shows that the conjugate concentration increased to a maximum in 12 to 14 hours after exposure and then dropped rapidly for the next 4 hours.

Obviously, elimination of metabolized Sevin is a rapid phenomenon. Thus, accumulation of Sevin in the animal seems unlikely under any normal condition of exposure.

These data suggest that a portion of the Sevin ingested by the cow is rapidly hydrolyzed to 1-naphthol which, in turn is detoxified to 1-naphthyl sulfate and eliminated in the urine. A trace of 1-naphthol or Sevin or both escapes detoxification and is eliminated free in the urine.

Analysis of Feces. Qualitative analysis of some of the spot samples collected in the Cornell experiment suggested that a small amount of Sevin was being eliminated unchanged in the feces. To check this point, a Hereford heifer was selected at a local farm and given a single dose of Sevin comparable in concentration to that received daily by the Cornell cows (approximately 10 p.p.m. on body weight). Feces samples were collected as soon as possible after elimination and frozen immediately in an atmosphere of carbon dioxide to prevent air contamination.

In the supplementary experiment, a good deal of care was taken to prevent exposure of the feces to air, thus protecting the residue. Table IV shows that, in this case, Sevin began to be eliminated 13 hours after exposure and reached a maximum in 16 to 19 hours. In 36 hours, only a trace was detected in the feces.

Upon differentiation, it was shown that the residue determined was Sevin and that only a trace of 1-naphthol was present. Conjugates of 1-naphthol were not present.

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