Heptachlor and Heptachlor Epoxide Residues on Fall-Treated Alfalfa and in Milk and Cow Tissues

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Heptachlor and heptachlor epoxide residues in trace amounts occurred in alfalfa harvested from fields treated the previous fall at 0.25, 0.50, and 1.00 pound of heptachlor per acre. Heptachlor epoxide residues subsequently appeared in the fat tissue and milk of cows that ingested the treated hay over a period of 30 days. The highest concentration of residue in milk occurred between the 18th and 24th days of the feeding period and averaged 13, 26, and 49 p.p.b. for the test animals in each group. Removal of treated hay from the diet resulted in a sharp decline of residues in the milk, followed by a gradual disappearance of residue over 13 weeks. Analysis of tissues from animals sacrificed at the end of the investigation indicated minute traces of heptachlor epoxide still stored in the fat. The amount of heptachlor epoxide excreted in the milk and stored in the fat tissue was directly related to the quantity of heptachlor and heptachlor epoxide ingested in the diet.

The profitable production of alfalfa in the eastern and midwestern states is seriously threatened by the presence of the alfalfa weevil. Heptachlor applied as a granular formulation in the fall is an effective, convenient, and economical control for this pest.

However, heptachlor is a persistent organochlorine insecticide that is translocated from the soil into certain crops, including alfalfa (Bruce and Decker, 1966; Engel *et al.*, 1965; Lichtenstein and Schulz, 1965), and subsequently stored in the fat of dairy cows fed the treated hay (Bruce *et al.*, 1965; Rusoff *et al.*, 1963). Heptachlor epoxide residues are excreted in the milk of such animals for an extended period of time (Bache *et al.*, 1960; Bruce *et al.*, 1965; Davidow *et al.*, 1953; Gannon *et al.*, 1959; Huber and Bishop, 1962; Rusoff *et al.*, 1962; Westlake *et al.*, 1963). Because of the presence of heptachlor epoxide residues in milk following heptachlor treatment of alfalfa, the registration for such use was withdrawn in 1964 by the Pesticides Regulation Division, U. S. Department of Agriculture.

Previous investigations conducted in Ohio (Ware, 1964) have raised questions about the validity of relating residue data from controlled feeding studies using artificial methods of administration to the actual condition where the alfalfa hay in the diet came from fields treated the previous fall. Huber and Bishop (1962) conducted heptachlor and heptachlor epoxide residue studies on alfalfa and milk following spring treatment of alfalfa at 2.0 pounds per acre of heptachlor. Preliminary residue investigations in Ohio (Ware, 1964) showed only six of 50 hay samples treated by the farmer the previous fall at 1.0 pound per acre to have detectable heptachlor epoxide residues, ranging from 0.01 to 0.1 p.p.m. Only one of the subsequent 1500 milk samples analyzed by electron-capture gas chromatography was contaminated at 0.007 p.p.m. of heptachlor epoxide.

The purpose of this investigation was to determine the extent of heptachlor and heptachlor epoxide residues occurring in alfalfa, and subsequently in milk and cow tissues, from fall treatments of various levels of granular heptachlor.

EXPERIMENTAL PROCEDURE

Treatment and Sampling of Alfalfa. Granules containing 2.5% heptachlor on 20- to 40-mesh Attaclay were applied November 2, 1965, to three plots of alfalfa at the South Charleston, Ohio, Agricultural Research and Development Center Branch Farm at the rate of 0.25, 0.50, and 1.00 pound of insecticide per acre. Green alfalfa samples were collected by hand from the three treated plots and a check plot on May 3, 10, 17, 24, and 30, 1966. Samples of soil and of alfalfa crowns and roots were collected May 24 from the same sampling sites. The soil was air-dried and the severed crowns and roots were washed thoroughly to remove the soil. Leaves were clipped and separated from the stems on portions of the alfalfa samples collected on May 10 and 30. All alfalfa samples were chopped in a Hobart food chopper, frozen, and stored at $-20°$ C. until time of analysis.

The treated fields were cut on June 1, 1966, and the hay was baled and tagged for the appropriate plot. Each bale of treated hay was sampled using a Pennsylvania hay sampler at the time of feeding. The cores from similar treatments were composited at about 10-day intervals during the feeding study, and analyzed for residue. Heptachlor and heptachlor epoxide residues in green alfalfa, hay, and soil were determined by a slight modification of a

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Velsicol Chemical Corp. method (1964), using acetonitrile extraction, pentane partitioning from aqueous solution, Florisil column cleanup, and electron-capture gas chromatographic measurement on a 4-foot glass column of 3.8% SE-30 on 80- to 100-mesh Diataport S at a column temperature of 190° C. and detector temperature of 200° C.

Heptachlor Epoxide Residues in Milk. After the milk of several cows had been screened for the heptachlor epoxide background or other interfering constituents, 12 Holstein cows were purchased on the open market and housed at the Waterman barn on The Ohio State University farm. The animals were placed on a standard ration for 2 weeks while getting accustomed to the new environment. Milk samples were collected and analyzed during this period to provide a control base for future analyses. The hay, grain, and water fed during this time and the straw used for bedding were also analyzed to detect possible extraneous routes of contamination. The analysis of feeds and straw continued intermittently during the entire investigation.

The animals were divided into four groups of three cows each to receive diets containing hay from one of the following treatments: (1) 0.00, (2) 0.25, (3) 0.50, and (4) 1.00 pound of heptachlor per acre. The cows were fed these diets for 30 days and then all reverted to untreated diets for an additional 91 days. The amount of treated hay consumed daily by each cow was recorded, as well as the amount of milk produced. Eight-ounce milk samples from the evening and the following morning milking periods were collected and combined for each cow at 0, 2, 7, 12, 18, 24, and 30 days during the feeding trial. Milk samples were collected on the seventh day after treated hay was withdrawn, and then weekly until termination of the experiment. Samples were stored in the freezer when they could not be analyzed within 1 to 2 days from the time of collection.

Heptachlor and heptachlor epoxide residues were determined in each milk sample according to the method outlined by the Velsicol Chemical Corp. (1964), utilizing saponification of the fat in the whole milk and gas chromatographic determination. The sulfuric acid cleanup procedure was not employed. The chromatographic columns used were a 4-foot column of 3.8% SE-30 on 80- to 100-mesh Diataport S at a temperature of 190° C. with the electron-capture detector on an F & M 400 Biomedical gas chromatograph and a 6-foot column of 1.5% SE-52 on Anachrom ABS at a temperature of 200° C. with the electron-capture detector on a Barber-Colman Series 5000 gas chromatograph.

Heptachlor and Heptachlor Epoxide Residues in Body Tissues of Cows. At the termination of the study, which extended for 91 days following the withdrawal of the cows from the treated hay, one cow from each group was sacrificed. Muscle tissue from the loin, fat tissue from the kidney, and liver tissues were analyzed for heptachlor and heptachlor epoxide residues by methods similar to that used for the analysis of green alfalfa, except as described below. Fifty grams of ground muscle or liver tissue was extracted with 200 ml. of 1 to 1 acetone-acetonitrile solution. The residue in the extract was partitioned into pentane from 800 ml. of 2% aqueous Na_2SO_4 solution as outlined in the procedure. The aqueous phase was extracted a second time with an additional 100 ml. of pentane. The first pentane phase was concentrated to a 10- to 20-ml. volume and then transferred to a 20 × 300 mm. reservoired column of 15 grams of activated Florisil topped with anhydrous sodium sulfate. The second pentane solution was used to elute the column. After this elution, another receiving flask was used and the column was eluted with 150 ml. of 7.5% diethyl ether in pentane. The pentane eluate contained the heptachlor, whereas the elution of heptachlor epoxide required the ether solution. The eluates were concentrated with a vacuum evaporator to an appropriate volume and aliquots were injected on the gas chromatographic columns at conditions specified above.

Twenty-five grams of fat was blended for 2 to 3 minutes with 100 ml. of acetone. Then 100 ml. of acetonitrile was added, which coagulated the fat, and the blending continued for an additional 2 to 3 minutes. The supernatant extract was chilled in a stoppered bottle in the freezer for 3 to 4 hours or overnight and then filtered while cold. After warming the filtrate to room temperature, a 100-ml. aliquot was analyzed by continuing the procedure used for muscle and liver tissue. The amount of Florisil was doubled to compensate for the residual fat still left in the extract.

RESULTS AND DISCUSSION

Heptachlor and heptachlor epoxide residues found in the green alfalfa at five sampling dates prior to cutting are listed in Table I. The low concentrations of heptachlor and heptachlor epoxide residue in green alfalfa samples appeared to be directly related to the level of alfalfa treatment and the concentration of residue appeared to be consistent during the 30-day sampling period. Thus, although as the plant grew larger it contained more total residues, the residue per plant weight basis remained constant. The ratio of heptachlor to heptachlor epoxide residue in the samples during this growth period shows slight metabolism of heptachlor to its epoxide in the plant. Analysis of equal weights of leaf and stem tissue from this growth period indicated that heptachlor and heptachlor epoxide were equally distributed throughout the edible portions of the plant and not concentrated in any particular tissue. Analysis of the whole alfalfa plant and the soil in which it grew resulted in a comparison similar to that reported by King *et al.* (1966). The largest concentration of residue occurred in the alfalfa crowns associated with the soil surface. Residues in the edible portions of the plant, as well as the roots, were small in comparison with that of the crown area. Consequently, the plant apparently absorbs the heptachlor through the root hairs located near the crown area and subsequently translocates it to other tissues. King *et al.* (1966) also found that heptachlor applied as granules remained near the soil surface and did not leach deeply into undisturbed soils. This latter characteristic is undoubtedly the basis for the theory that the majority of heptachlor residue in field-cured hay was attributed to dust during hay making. However, the results of this study, as well as those reported by King *et al.* (1966), indicate that heptachlor and its epoxide are translocated, although sometimes in only trace amounts, from the soil surface area to other parts of the plant.

Heptachlor and heptachlor epoxide residues found in the hay from the treated plots (Table I) showed basically the same type relationship of concentration to level of treatment that appeared earlier in the green alfalfa. However, on the basis of determined moisture content differences in green alfalfa and its subsequent dry hay, heptachlor and the epoxide residues were reduced 55 to 65% during field curing of the hay. This finding supports claims made by the Velsicol Chemical Co. and confirmed by King *et al.* (1966). At the present time, there is no satisfactory explanation of this phenomenon.

All of the cows included in this investigation maintained good health, body weight, and milk production throughout the investigation, except two cows (17 and 88) which contracted mastitis near the termination of the disappearance period. This affected only the milk production and had little significance on the entire investigation. Table II shows that the consumption of treated hay was approximately equal for all animals, averaging 20.156 kg. per day. However, hay was not wasted, because of the limited amount available. In spite of this restriction, at no time during the experimental period were weighbacks nonexistent. The daily hay ration supplemented by the normal grain ration for lactating cows was adequate, as indicated by the maintaining of the daily milk production (Table II).

Traces of heptachlor epoxide residue were found in the milk of all cows that were fed a diet containing alfalfa hay from fields treated with low concentrations of heptachlor the previous fall (Tables III and IV). The data in Table III show the residues for each test animal on the date of sampling. Figure 1 graphically portrays the average residue levels for the animals of each treatment. The data in Table IV show the average daily consumption of heptachlor and heptachlor epoxide and the excretion of heptachlor epoxide in milk. The average daily consumption was calculated from the average residue levels of dry hay for the various treatments reported in Table I related to the weight of hay consumed on the sampling day (Table II). The excretion in milk was calculated from the residue levels reported in Table III related to the average weights of milk produced on the samping day (Table II). Cows fed hay from the control and plots treated with 0.25, 0.50, and 1.00 pound of insecticide per acre ingested an average of 0.000, 0.3624, 0.8881, and 1.4665 μg., respectively, daily of heptachlor and its epoxide during the 30-day feeding period. Daily excretion of heptachlor epoxide in milk averaged 0.018 μg. apparent in the control and 0.118, 0.306, and 0.552 μg., adjusted to the control base line, for the respective treatments.

The data in Table III indicate that maximum levels of heptachlor epoxide residues occurred in the milk at ap-

Table I. Heptachlor and Heptachlor Epoxide Residues in Alfalfa Following Fall Applications of Granular Heptachlor

Sampling Dates	Rate of Application, Lb./Acre[a]	Residue, P.P.B. Heptachlor	Residue, P.P.B. Heptachlor epoxide
	GREEN ALFALFA		
5-3-66	0.25	5.0	14.9
	0.50	15.9	43.6
	1.00	35.2	64.5
5-10-66	0.25	5.3	16.9
	0.50	11.9	30.8
	1.00	27.3	43.7
5-17-66	0.25	7.6	16.7
	0.50	14.8	32.0
	1.00	22.2	60.7
5-24-66	0.25	5.6	25.7
	0.50	3.8	48.8
	1.00	8.7	64.1
5-30-66	0.25	6.9	20.3
	0.50	7.3	30.8
	1.00	20.1	71.6
	DRY HAY		
6-20-66	0.25	8.4	29.0
	0.50	22.7	62.3
	1.00	43.6	95.1
7-6-66	0.25	14.1	45.3
	0.50	21.7	57.4
	1.00	42.1	99.4
7-19-66	0.25	7.4	31.2
	0.50	21.3	72.7
	1.00	56.6	143.7

[a] Granules containing 2.5% heptachlor on 20- to 40-mesh Attaclay applied Nov. 2, 1965.

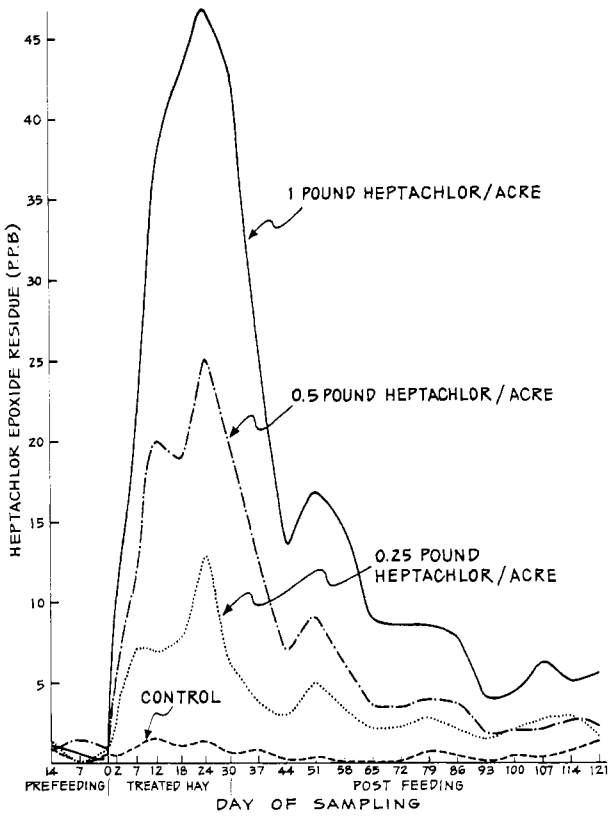

Figure 1. Accumulation and subsequent disappearance of heptachlor epoxide residue in milk of cows fed hay from fall-treated alfalfa fields

Mean values for three cows in each treatment

Table II. Average Daily Ingestion of Treated Hay and Daily Milk Production[a]

	Treatment Level of Heptachlor, Lb./Acre							
	Control		0.25		0.50		1.00	
Day of Sampling	Hay ingested, kg.	Milk produced, kg.	Hay ingested, kg.	Milk produced, kg.	Hay ingested, kg.	Milk produced, kg.	Hay ingested, kg.	Milk produced, kg.
0	12.22	19.69	8.59	18.73	9.87	20.91	8.48	18.42
2	9.01	19.99	7.65	20.52	8.98	23.69	8.63	20.55
7	9.01	20.25	8.30	20.81	8.68	22.02	8.30	19.82
12	9.89	20.25	8.27	18.04	9.45	23.26	9.72	18.96
18	9.37	18.90	7.35	19.16	9.19	20.97	8.57	18.60
24	12.34	19.10	7.94	19.34	12.11	23.77	10.25	18.75
30	11.31	20.44	8.68	19.20	11.66	21.29	9.66	19.70
Av.[b]	10.46	19.80	8.11	19.33	9.98	22.27	9.09	19.28

[a] Average of three cows in each treatment on sampling day.
[b] Average daily ingestion and milk production for test animals over 30-day feeding period of treated hay.

Table III. Heptachlor Epoxide Residues in Milk of Dairy Cows Fed Hay from Fall-Treated[a] Alfalfa Fields

Data corrected for base of 3.5% butterfat milk

Date 1966			Heptachlor Epoxide Residues for Cows by Treatment and Number[b], P.P.B.										
			Untreated Hay			0.25 Lb./Acre			0.50 Lb./Acre			1.00 Lb./Acre	
Mo.	Da.	Sampling Description	93	04	26	14	17	54	43	68	08	87	88	12
6	3–4	Init. prefeed.	0.00	0.00	2.40	1.65	0.00	2.65	2.65	0.00	0.00	0.72	0.00	2.38
6	10–11	1-wk. prefeed.	0.00	0.00	0.00	0.00	0.00	0.00	2.73	0.00	1.49	1.05	0.46	0.30
6	17–18	2-wk. prefeed., 0-day	0.34	0.32	1.31	2.01	0.00	0.48	0.88	2.09	0.00	0.00	0.00	1.11
6	19–20	2-day trtd. feed	0.12	0.03	1.03	1.45	3.18	2.84	5.44	4.44	7.42	8.19	8.62	11.60
6	24–25	7-day trtd. feed	0.73	1.07	1.55	5.67	9.31	6.56	6.36	10.58	17.85	18.94	19.03	23.57
6	29–30	12-day trtd. feed	1.52	0.26	3.08	8.32	6.10	5.29	26.00	16.49	17.50	31.65	45.40	35.05
7	6–7	18-day trtd. feed	0.39	0.29	2.46	11.30	6.43	5.57	16.58	18.66	21.49	44.50	42.60	41.10
7	12–13	24-day trtd. feed	1.33	2.24	0.87	10.37	17.03	9.96	21.68	24.66	28.80	40.60	45.54	54.40
7	18–19	30-day trtd. feed	0.72	0.23	0.92	7.25	3.59	7.71	15.98	23.32	21.42	41.48	45.70	42.80
7	25–26	1-wk postfd.	1.28	1.10	0.18	4.51	2.56	4.41	8.89	14.10	14.73	23.84	22.68	26.24
8	1–2	2 wk. postfd.	0.07	0.44	0.00	4.06	2.33	2.71	5.83	6.65	8.63	14.92	10.48	16.22
8	8–9	3-wk. postfd.	0.54	0.15	0.37	4.74	6.03	4.33	7.69	6.69	12.79	16.10	13.82	20.39
8	15–16	4-wk. postfd.	0.10	0.10	0.00	5.10	2.55	2.73	6.80	6.95	5.35	19.28	10.31	15.08
8	22–23	5-wk. postfd.	0.19	0.00	0.14	2.96	1.44	1.41	2.44	5.44	5.13	10.70	7.50	8.42
8	29–30	6-wk. postfd.	0.00	0.00	0.36	2.47	2.21	2.19	4.81	3.32	2.48	11.26	7.61	7.13
9	5–6	7-wk. postfd.	0.91	0.59	0.79	3.40	[c]	2.32	5.13	3.81	2.91	11.48	6.16	8.36
9	12–13	8-wk. postfd.	0.28	0.50	0.85	2.02	[c]	2.43	5.24	3.44	2.59	8.55	8.17	7.18
9	19–20	9-wk. postfd.	0.00	0.00	0.00	1.73	[c]	1.48	2.08	2.16	1.13	5.24	c	2.70
9	26–27	10-wk. postfd.	0.77	0.49	0.42	1.71	3.43	2.31	3.52	1.66	0.84	5.35	14.68	3.37
10	3–4	11-wk. postfd.	1.33	0.00	0.00	2.84	4.68	2.65	3.93	2.71	0.00	7.95	13.48	4.62
10	17–18	13 wk. postfd.	3.30	0.26	0.48	2.05	4.55	1.74	3.64	2.27	0.87	6.94	5.81	4.07

[a] Granules containing 2.5% heptachlor on 20- to 40-mesh Attaclay.
[b] Number represents last two digits of ear tag.
[c] Mastitis.

Table IV. Average Daily Excretion of Heptachlor Epoxide in Milk in Relation to Average Daily Ingestion of Heptachlor by Test Cows[a]

	Average Residues[a] on Sampling Day according to Treatment Level of Heptachlor, Lb./Acre							
	Control		0.25		0.50		1.00	
Day of Sampling	Heptachlor ingestion, μg.	Hept. epox. excretion, μg.	Heptachlor ingestion, μg.	Hept. epox. excretion, μg.	Heptachlor ingestion, μg.	Hept. epox. excretion, μg.	Heptachlor ingestion, μg.	Hept. epox. excretion, μg.
0	0.000	0.0114	0.000	0.0129	0.000	0.0180	0.000	0.0122
2	0.000	0.0086	0.3452	0.0437	0.7966	0.1165	1.3828	0.1637
7	0.000	0.0212	0.3746	0.1270	0.7698	0.2225	1.3294	0.3503
12	0.000	0.0301	0.3732	0.0979	0.8382	0.3334	1.5770	0.6066
18	0.000	0.0219	0.3316	0.1356	0.8154	0.3278	1.3730	0.7490
24	0.000	0.0235	0.3582	0.2071	1.0742	0.4746	1.6418	0.6817
30	0.000	0.0092	0.3916	0.0946	1.0342	0.3588	1.4946	0.7612
Av.[b]	0.000	0.0181	0.3624	0.1176	0.8881	0.3056	1.4665	0.5521

[a] Residues recorded are averages from three cows in each treatment on sampling day while on diets containing treated hay.
[b] Residues represent average daily ingestion and excretion of three cows in each treatment over 30-day treatment period excluding 0 day where milk sampling occurred before ingestion of treated hay.

proximately the 18th to the 24th day of feeding treated hay. Residues declined rapidly during the first 2 weeks after withdrawal of the treated hay, then gradually over the remaining part of the experiment. At 13 weeks postfeeding traces of residue were still found in the milk of cows fed hay from the 1 pound per acre treatment, whereas the milk of all others approached or had attained the control level. Cows 17 and 88 contracted mastitis. At the time they recovered and began to produce milk again, a noticeable increase in heptachlor epoxide residue occurred. Perhaps this may be attributed to the stress caused by the disease accompanied by a drastic reduction in milk production.

The data show a direct relationship between heptachlor and heptachlor epoxide concentrations in the hay (which corresponded to application rates) consumed by the cows and those appearing in the milk. The average of the high residue values of the three cows in each group while on the diet including treated hay (Figure 1) showed that heptachlor epoxide residues reached a concentration in the milk of 13 p.p.b. at 0.25 pound per acre, 26 p.p.b. at 0.50 pound per acre, and 49 p.p.b. at 1.00 pound per acre. The daily excretion in the milk approximated one third of the daily ingestion during the feeding period. The fate of the remaining two thirds of the ingested residue was not quantitatively ascertained. Undoubtedly (Bush *et al.*, 1966), some was excreted in waste materials and a substantial amount was stored in the fat tissues of the animal.

The results of the tissue residue analysis of the four animals sacrificed are presented in Table V. The data show that little or no residue was present in the muscle tissue but minute traces of heptachlor epoxide were present in the liver of all treated animals. The fat of all treated animals contained residues of heptachlor epoxide at concentration levels proportional to the amount ingested. However, no attempt was made to quantitate the total amount of residue present in the body fat of the animal after the treatment period or at the time of slaughter. The results show only that heptachlor epoxide was stored in the fat, was released into the butterfat of the milk over a period of time, and some residue still was present in the fat at the time the animals were sacrificed. The cause of apparent residues in the muscle and fat tissue of the control animal is not known. Examination of her liver after the sacrificing indicated possible disease, difficulty, or contamination in her earlier history that may have produced artifacts which affected the tissue analysis.

Table V. Heptachlor and Heptachlor Epoxide Residues in Cow Tissues after 30-Day Feeding Period and 91-Day Postfeeding Period

Cow No.	Hay from Fall Treatment, Lb./Acre	Tissue	Residues, P.P.B.[a] Heptachlor	Heptachlor epoxide
93	Control	Muscle	1.91	3.85
		Liver	0.00	0.00
		Fat	4.13	0.97
17	0.25	Muscle	0.00	0.00
		Liver	0.00	3.39
		Fat	0.00	40.8
43	0.50	Muscle	0.00	1.99
		Liver	0.00	11.12
		Fat	0.06	63.3
87	1.00	Muscle	0.00	0.00
		Liver	0.00	15.7
		Fat	2.40	88.1

[a] Residues for cows 17, 43, and 87 corrected for apparent residue in control cow 93, which in turn was corrected for interferences experienced in methods.

The results of this investigation show that heptachlor and heptachlor epoxide residues in trace amounts occur in alfalfa harvested from fields treated the previous fall with low concentrations of granular heptachlor formulations. The residue ingested by the cow subsequently appears in the fat tissues and in the milk. However, the concentration of residue in the milk was very low, the highest reported value being 0.054 p.p.m. which occurred during the feeding of treated hay. The residue stored in the fat gradually declined over a considerable period of time. It was not determined how long measurable residues might persist in body fat or whether they would appear from time to time in later milk history. Although the results under the conditions of this study indicate that the residues in milk occurring from the slow release of trace amounts of heptachlor epoxide from the fat would be insignificant, they show that under normal conditions heptachlor epoxide cannot be rapidly flushed from the fat of a contaminated animal.

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Received for review January 24, 1968. Accepted April 4, 1968. Research partially supported by the Velsicol Chemical Corp. in State Special Grant Number 147.