Fate of *S*-[(2-Methoxy-5-oxo-Δ^2-1,3,4-thiadiazolin-4-yl)methyl] *O,O*-dimethyl phosphorodithioate (Supracide) in a Lactating Cow

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A cow was fed C^{14}-labeled *S*-[(2-methoxy-5-oxo-Δ^2-1,3,4-thiadiazolin-4-yl)methyl] *O,O*-dimethyl phosphorodithioate (Supracide) for 5 days at the rate of 1 mg. per kg. per day. No Supracide or its oxygen analog was found in the milk. The sensitivity of the analytical procedures was 0.01 p.p.m. for each compound. The total amount of radioactivity found in the milk during the 15-day study represented only 0.6% of the oral dosage. Fractionation of the milk indicated extensive metabolism, as did the nature of the radioactivity recovered in the urine (24%) and feces (34%). Total radioactivity determined in tissues indicates that significant storage does not occur. The highest level of radioactivity found in tissues was 0.11 p.p.m. in the liver.

Supracide, a new organophosphorus insecticide having the chemical structure *S*-[(2-methoxy-5-oxo-Δ^2-1,3,4-thiadiazolin-4-yl)methyl] *O,O*-dimethyl phosphorodithioate, referred to as GS-13005, effectively controls the alfalfa weevil. Since alfalfa is such an important forage crop for the dairy industry, the amount and nature of residues which might be found in milk from a cow fed this pesticide are of prime concern in the evaluation of its use on forage. A secondary concern is the amount of pesticide or its derivatives that might be present in the vital organs, edible tissues, and various fat deposits of cattle following ingestion of this pesticide.

This paper gives the results of an investigation of the amounts and nature of residues in milk, tissues, blood, urine, and feces following administration of 5-carbonyl-C^{14} GS-13005 to a cow at the rate of 1 mg. per kg. per day over a 5-day period. By including the analyses of blood, urine, and feces in this investigation, the extent of the metabolism of GS-13005 by a cow could be evaluated.

EXPERIMENTAL METHODS

Treatment and Sampling. Two Holstein dairy cows of average milk production and in good physical condition, weighing 500 kg. (treated) and 455 kg. (control), respectively, were provided by free choice 14 pounds of Purina dairy chow (16% protein) and 20 pounds of mixed hay daily. After preconditioning in metabolism stalls, the control cow received daily three capsules containing corn starch by means of a balling gun, whereas the treated cow received capsules containing corn starch and 164.7 mg. of 5-carbonyl-C^{14} GS-13005 (6.67 μc. per mg.) (Cassidy *et al.*, 1969), this feeding rate being equivalent to 1 mg. per kg. per day. The schedule consisted of a 5-day pretreatment, a 5-day treatment, and a 10-day post-treatment period.

Milk was collected twice a day using milking machines. Gooch tubing was gusseted between two 4-inch squares of rubber sheeting which were cemented around the cow's vulva so that the urine and feces could be collected separately. Excreta were taken daily and subsampled. Once a day, blood samples were taken and heparin was added, this operation being carried out in the afternoon 1 hour after the second capsule was given. All samples were kept frozen until analyzed. At the end of the post-treatment period, both cows were slaughtered. The following tissues were subsampled and frozen: brain, heart, kidney, liver, round muscle, tenderloin muscle, spleen, blood, and fat (omental, tail head, perirenal, and subcutaneous).

Radioactive Techniques. All counting was done with a Packard Tri-Carb scintillation spectrometer, Model 3365. Urine samples were counted directly in Bray's solution (1960). Feces samples were extracted with acetone-methanol (1 to 1), the extracts concentrated to known volumes, and aliquots taken for direct counting in Bray's solution. All counts were corrected using an internal standard. The insoluble fecal residues were counted after Schöniger combustion, as described by Kalberer and Ruthschmann (1961). Blood samples were counted after a Schöniger combustion and/or wet combustion (Jeffay and Alvarez, 1961; Van Slyke and Folch, 1940).

Whole milk samples were counted, using a method similar to that described by Steinberg (1960). Five milliliters of milk containing 10 μl. of a wetting reagent (Alrowet 50) was pipeted into 2.66 grams of anthracene and mixed. A calibration curve was prepared using 5-carbonyl-C^{14} GS-13005 as a standard. The range was linear for a content from 0.054 to 2.5 μg. in milk, the counting efficiency being 15.3%.

A thin-layer chromatographic method was used to detect picogram amounts of the oxygen analog of GS-13005 in whole milk (Mattson *et al.*, 1969). A 10-μl. aliquot of whole milk was spotted on a silica gel layer and developed with chloroform-acetone (9 to 1). Detection was by inhibition of flyhead cholinesterases. If the oxygen analog were present, it would be detected as a white spot on a colored background.

Milk samples were fractionated as described by Adams and Anderson (1966). The scheme is shown in Figure 1. Direct counting with internal standardization using either toluene or Bray's scintillation liquids was employed in the case of the benzene and water-soluble fractions of whole milk. The radioactivity in the milk insolubles was determined both by Schöniger and Van Slyke combustions.

Total radioactivity in the tissues, except for the fats, was determined by the Schöniger technique, using a 3-liter flask and 0.5 gram samples. Recovery data for 0.5-gram samples injected with 5-carbonyl-C^{14} GS-13005 at the 0.02 and 0.10 p.p.m. levels were excellent using

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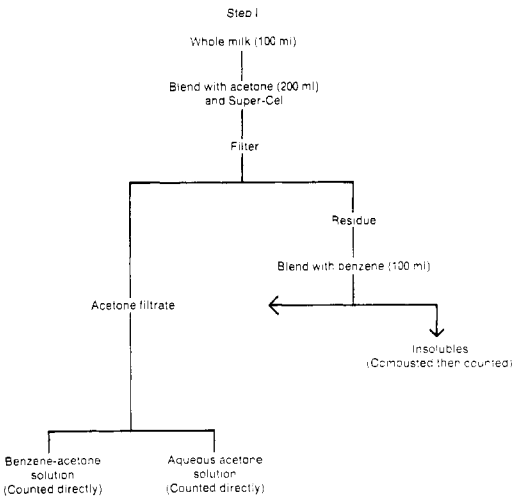

Figure 1. Fractionation of milk into three parts

seven different types of tissue, 93% (S.D. ± 6%) at the low level and 93% (S.D. ± 2%) at the high level. About 500 grams of each tissue was ground while frozen and six 0.5-gram samples were taken at random for analysis. A count twice background was equivalent to 0.01 p.p.m. of this radioactive pesticide.

Total radioactivity in fats was determined by pretreating 0.5-gram samples which had been finely chopped with 4 ml. of an NCS toluene quaternary ammonium base (Hansen and Bush, 1967). The samples were heated in closed vials on the steam bath for several hours, cooled, diluted to 20 ml. with toluene scintillation liquid, and counted.

Gas Chromatographic Method. As little as 0.01 p.p.m. of GS-13005 or its oxygen analog can be detected in milk. Four steps are necessary: I, an extraction, as shown in Figure 1; II and III, a partition clean-up and a column chromatographic clean-up, as shown in Figure 2; and IV, a final analysis by gas chromatography. An aliquot equivalent to 10 grams of milk can be injected into the gas chromatograph using this method.

The instrument was a Micro Tek Model 220 GLC equipped with a Dohrmann C-200 microcoulometer and a sulfur detection cell No. T-300. A 4-foot × ¼-inch O.D. 7740 glass column packed with 3% SE 30 and 0.3% EPON 1001 on Gas Chrom Q was used with a nitrogen carrier flow of 80 ml. per minute, a nitrogen purge of 20 ml. per minute, and an oxygen flow through the furnace of 50 ml. per minute. The injector, column, transfer, and furnace temperatures were 225°, 190°, 250°, and 875° C., respectively. Under these conditions the retention time of GS-13005 and the oxygen analog were 4.8 and 5.5 minutes; minimum detectabilities, respectively, were 10 and 50 nanograms. A standard curve was prepared by injecting known amounts and determining peak areas. Recoveries of 0.01 p.p.m. of pesticide added to milk ranged from 67 to 90% for GS-13005 and from 58 to 78% for the oxygen analog.

Two main difficulties encountered in developing this method involved the oxygen analog. Standardization of each batch of Florisil was necessary. Of the many gas chromatographic columns evaluated, only the one described gave usable elution characteristics; the addition of EPON 1001 is the critical factor. Figure 3 shows tracings of typical chromatograms of column effluents for aliquots of milk samples carried through steps I to III. The microcoulometric sulfur detector was used because of its comparative specificity. An electron-capture detector, which was used for screening selected samples for other residues, could have been used; however, it was not as specific as the microcoulometric detector.

RESULTS AND DISCUSSION

Total Radioactivity in Urine and Feces. About 24% of the total radioactivity ingested by the cow was re-

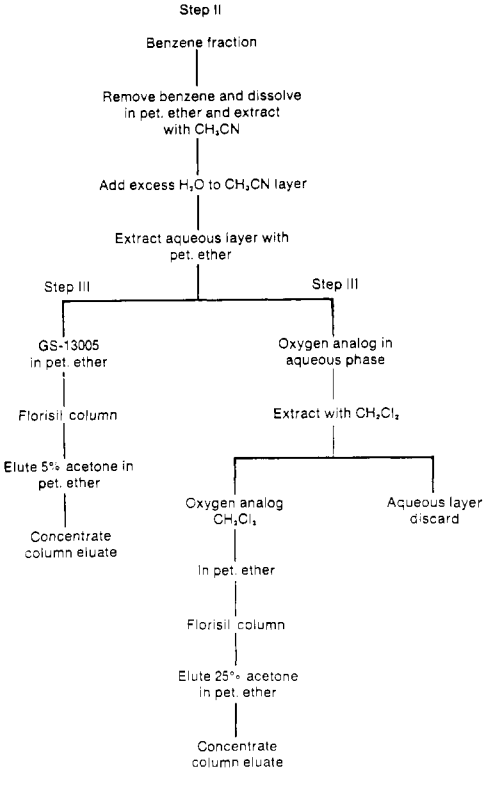

Figure 2. Partition and column clean-up of benzene-acetone fraction of milk

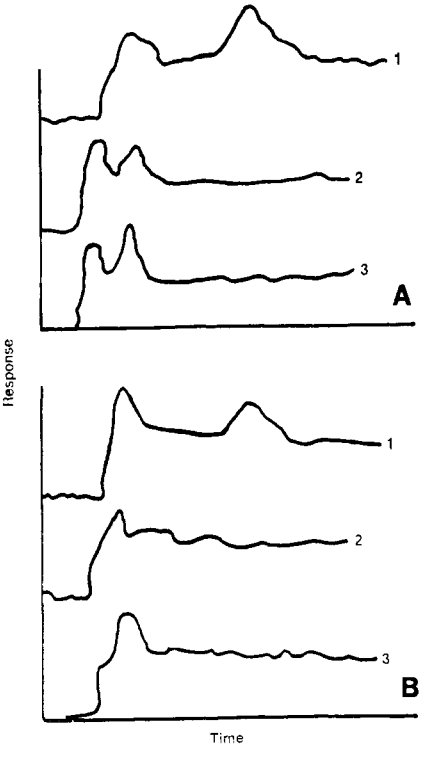

Figure 3. Typical microcoulometric chromatograms of GS-13005 column eluates (A) and of oxygen analog eluates (B) of milk samples

1. Milk fortified with 0.01 p.p.m.
2. Milk from treated cow
3. Milk from control cow

covered in the urine. The radioactivity did not plateau but peaked, the largest amount being in the specimen collected on the third day of feeding. After the last capsule was administered, the radioactivity decreased. The urine data are shown in Figure 4. A paper chromatogram developed with an acetonitrile-water-ammonia (40:9:1) system indicated that 85% of the radioactivity in the urine specimen collected on the third day of treatment was due to a very polar metabolite with an R_f of 0.23. The R_f's of radioactive zones did not correspond to the R_f's for GS-13005, its oxygen analog, or 2-methoxy-Δ^2-1,3,4-thiadiazolin-5-one (designated RH), the limit of detectability being 0.15 p.p.m. equivalent to GS-13005. No further work was done with the urine.

About 34% of the total radioactivity ingested by the cow was recovered in the feces. Eighteen per cent was found in the acetone-methanol extracts and 16% in the fecal residues. The peak amount of radioactivity was found in the specimen collected on the fourth feeding day. This finding differs from that for the urine, the peak amount being found on the third day. After the last capsule was administered, the radioactivity fell rapidly. By the fourth post-treatment day, only trace amounts of radioactivity were found in the feces (Figure 4).

A two-dimensional thin-layer chromatogram, initially developed with chloroform-acetone (9 to 1) and then with acetonitrile-water-ammonia (40:9:1), indicated that the majority of the radioactivity in the fecal extract was due to polar metabolites. In the radioautogram, no zone corresponded to that for GS-13005, its oxygen analog, or RH, the limit of detectability being about 1 p.p.m. equivalent to GS-13005. The radioactivity in the residue must be due to very polar or bonded metabolites, because methanol and acetone are efficient extracting solvents.

Examination of the records shows that for each cow no essential change in the amounts of milk, urine, and feces occurred during the course of the study. The differences in excreta between cows were within normal limits (Dukes, 1955).

Total Radioactivity in the Blood. The radioactivity in the blood peaked the day the cow received her last capsule, the radioactivity being equivalent to 0.2 p.p.m. of GS-13005. Thereafter it fell rapidly; by the tenth post-treatment day it was only 0.02 p.p.m.

This radioactivity was determined by two combustion techniques. When the Van Slyke combustion technique was used, the thiadiazole ring (5-carbonyl-C^{14} RH) was not combusted quantitatively to $C^{14}O_2$; only about a 60% recovery was obtained. For the Schöniger technique, the combustion and recovery were quantitative. The data for the radioactivity found in the blood are presented in Figure 5.

By the second post-treatment day the values obtained by both techniques were the same; thus, the radioactivity found in the blood during the post-treatment period does not seem to be present as C^{14}-carbonyl in the thiadiazole ring.

Total Radioactivity in Milk. No oxygen analog of GS-13005 was detected in any of the milk samples by thin-layer chromatography using the flyhead homogenate spray as a means of detection (sensitivity 0.01 p.p.m.).

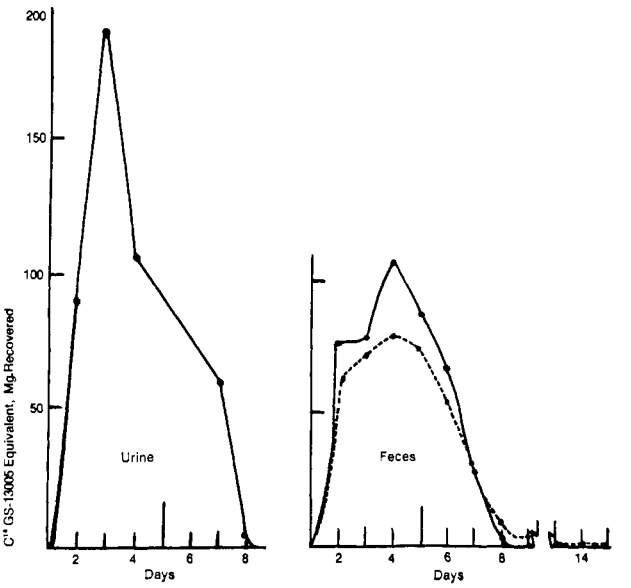

Figure 4. Excretion pattern and recovery of radioactivity in feces and urine
5th day—end of feeding period
Feces extracts ————
Feces residues - - - - - - -

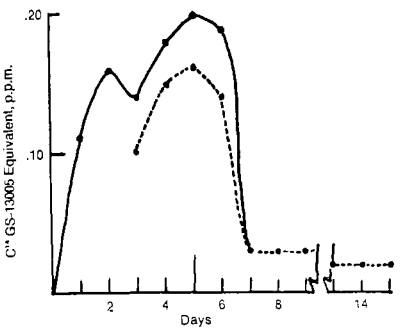

Figure 5. Radioactivity in blood
5th day—end of feeding period
Schöniger combustion —— 0-9 days
Wet combustion - - - - - - - - 0-15 days

Typical chromatograms showed darkish zones in the upper sections, but no evidence of a discrete white zone was noted in the zone area for the oxygen analog. Indeed, no evidence was found for any cholinesterase inhibitors in any sample except those fortified with GS-13007.

The radioactivity in the whole milk did not plateau, but peaked the day after the treated cow received her last capsule, the radioactivity being equivalent to 0.5 p.p.m. of GS-13005. Thereafter, the radioactivity in the milk fell rapidly, and by the end of the tenth post-treatment day it had fallen to an equivalent of 0.004 p.p.m. of GS-13005. Based on direct counting using anthracene as the fluor, 0.6% of the radioactivity given the cow was found in the milk. No differences could be detected between milk samples from the two cows during the pretreatment period (5% risk, counts per 10 minutes, $F'_{0.95} = \frac{S^2 \text{ treated cow}}{S^2 \text{ control cow}}$, $\sigma^2_t = \sigma^2_c$, if $F'_{0.95} < 2.98$, $F' = 1.14$). With a 99% confidence level, the apparent limit of detectability (Sutherland, 1965) of radioactivity is 184 counts per 10 minutes, i.e., this

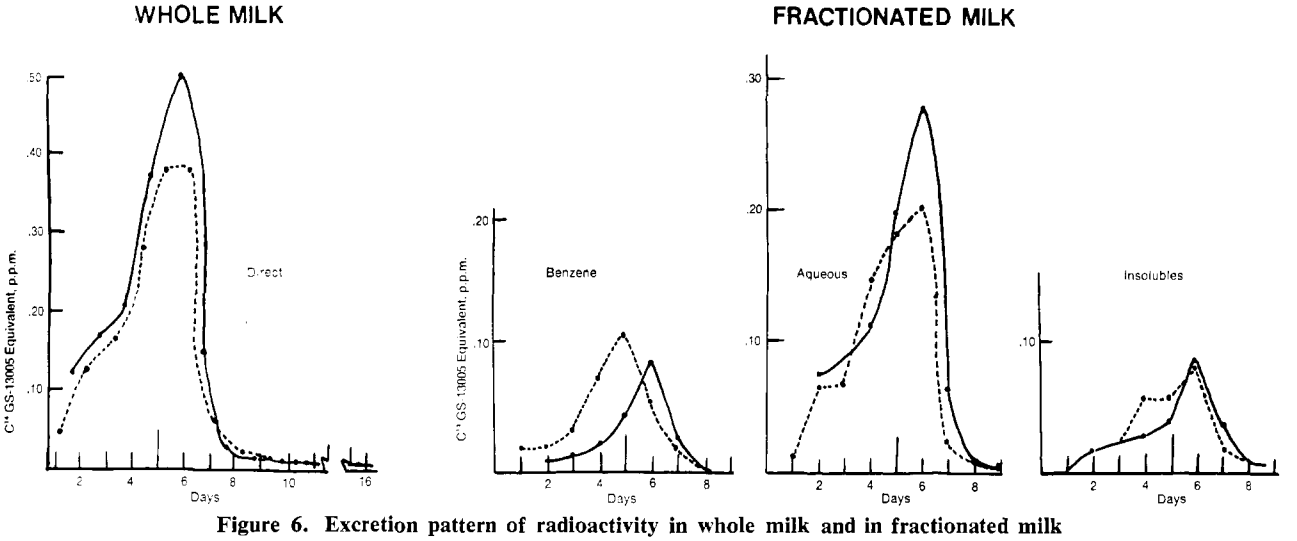

Figure 6. Excretion pattern of radioactivity in whole milk and in fractionated milk

5th day—end of feeding period AM ——— PM - - - - - - - -

count is the background level for the milk samples. A count of twice background (368 counts per 10 minutes) is equivalent to 0.002 p.p.m. of GS-13005. The data for the radioactivity found in whole milk samples are graphically presented in Figure 6.

Fractionation of Milk. To gain further insight into the nature of the radioactive metabolites in milk, all samples were fractionated into three parts, as shown in Figure 1. Three control milk samples were spiked separately with 5-carbonyl-C^{14} GS-13005, 5-carbonyl-C^{14} oxygen analog, and C^{14} sugar at 0.01 p.p.m.; 100% of the GS-13005 was found in the benzene fraction, 94% of the oxygen analog in the benzene fraction, and 6% in the aqueous fraction, 87% of the sugar was found in the aqueous fraction and 6% in the insoluble filter pad.

When the milk samples for the feeding period were fractionated in the same manner, more radioactivity was found in the afternoon benzene fraction of a milk sample than in the organic fraction of a milk sample of the following morning. These results indicate that considerable metabolism of this pesticide occurred overnight. The maximum amount of radioactivity in a benzene fraction equivalent to GS-13005 (0.107 p.p.m.) was found in the afternoon fraction of the milk sample taken the last day of feeding; in contrast, the radioactivity in the whole milk peaked the following morning. The data for radioactivity in benzene fractions are shown in Figure 6.

Considerable polar and insoluble metabolites were found in the aqueous and insoluble fractions. The radioactivity in the aqueous fractions was more than twice that in the benzene fractions. The maximum amount of radioactivity (0.28 p.p.m.) was found in the morning aqueous fraction of the milk sample taken the morning after the last feeding day. The radioactivity in the filter pads containing the insolubles also was the highest (0.086 p.p.m.) for the sample taken the day after the last capsule was given to the cow. The data for the aqueous fractions and those for the insolubles are shown in Figure 6. For aqueous fractions of milk samples during the feeding period, less radioactivity was found in an afternoon aqueous fraction of a milk sample than in the aqueous fraction of a milk sample taken the following morning, this being just the opposite of results for the benzene fractions. These results for the aqueous and insolubles fractions support the data for benzene fractions—i.e., considerable metabolism of this pesticide to polar metabolites occurred overnight.

The radioactivity in the filter pads was determined both by Schöniger and Van Slyke combustions. The results were the same by both techniques. As discussed earlier, when 5-carbonyl C^{14} GS-13005 or 5-carbonyl-C^{14} RH is oxidized using the Van Slyke solution, recoveries are only about 60%. Since no differences were found for the radioactivity in the filter pads by these two different techniques, the radioactive carbon in the insolubles must be due to metabolized GS-13005, with the carbon possibly incorporated in a large molecule, such as casein.

For milk samples taken around the end of the feeding period, about 70% of the radioactivity of the whole milk was in the two liquid fractions and 20% in the pads. However, during the last five days of the posttreatment period just over half of the radioactivity in the milk was in the aqueous fraction; and the rest was in the insolubles. These findings indicate extensive metabolism of GS-13005 and the possible incorporation of the radioactive carbon into natural products such as lactose.

For all milk samples having radioactivities greater than about 0.3 p.p.m. equivalent to GS-13005, the sums of the radioactivities in the corresponding benzene, aqueous, and insoluble fractions of a sample are about 90% of the radioactivity as determined for the milk sample using anthracene as the fluor.

If GS-13005 and its oxygen analog were present in the milk samples, they would be found in the benzene fractions. When milk samples were spiked at 0.01 p.p.m., the recoveries for 5-carbonyl-C^{14} GS-13005 and 5-carbonyl-C^{14} oxygen analog were 86 and 66%, respectively, by gas chromatography. In actual milk samples, no GS-13005 or its oxygen analog could be detected at this 0.01 p.p.m. level.

The radioactivities in the GS-13005 column eluates (Figure 2) used for column injection were never equivalent to 0.01 p.p.m. of GS-13005. These fractions

were chromatographed two-dimensionally by thin-layer chromatography and the chromatograms sprayed with sulfuric acid. In these fractions, lipids were detected in the zone area for GS-13005. Radioautograms of unsprayed chromatograms show radioactivity in the lipid zones.

Of the oxygen analog column eluates (Figure 2), only two contained radioactivity equivalent to about 0.01 p.p.m., these being obtained from afternoon milk samples taken on the fourth and fifth feeding days. Aliquots of the fifth day GS-13007 fraction were chromatographed on silica gel, and the thin-layer chromatograms sprayed with flyhead homogenate spray. Aliquots of raw whole milk for the fifth treatment day and the first post-treatment day were analyzed for anticholinesterase activity by an in vitro Hestrin method (Cook, 1954). If this radioactivity had represented GS-13007, enzymatic inhibition would have been observed; none was found.

Aliquots of the more radioactive oxygen analog fractions were also chromatographed two-dimensionally by thin-layer chromatography. These fractions were very clean, as was demonstrated by using silver nitrate and sulfuric acid sprays on the resulting chromatograms. Radioautograms of unsprayed chromatograms showed one discrete zone in the area for GS-13007. Although the zone area corresponded to that for the oxygen analog, this radioactive material could not represent this compound. If it had been, anticholinesterase activity would have been found in these fractions with radioactivity equivalent to 0.01 p.p.m.

All analytical data for these milk samples clearly indicate that less than 0.01 p.p.m. of GS-13005 or its oxygen analog was present in the milk of the treated cow and that most of the metabolites in milk represent polar metabolites, with evidence of destruction of the thiadiazole ring.

Total Radioactivity in Tissues. The levels of radioactivity in the tissues were low. The liver and kidney tissues had the highest level of radioactivity, equivalent to 0.11 and 0.04 p.p.m. of GS-13005, respectively. In the spleen, 0.03 p.p.m. was found, in the rest of the tissues, 0.02 p.p.m. In Table I are summarized the data for tissues of the treated cow, excluding the fats. All these values are low and are in the range that was expected—e.g., analogous to the work of Everett *et al.* (1966) on the nature and extent of Guthion residues in a cow. No attempt was made to determine whether the radioactive carbon was present as the parent compound, a primary and/or secondary metabolite.

The levels of radioactivity in the fats equivalent to GS-13005 were also low (0.02 to 0.03); the data are shown in Table II. Because of the difficulty of burning fat, the Nuclear Chicago NCS solubilizer was used to determine radioactivity in half-gram samples. The perirenal, subcutaneous, and tail head fats appeared to be completely in solution, with only a few very fine globules in the final solution used for counting. All these samples showed significant quenching due to a yellow color—i.e., 30 to 60%. Consequently, these values for the fats are not as reliable as those for the other tissues; however, they should be close to the correct values, even though this technique using a solubilizer must be refined for the analysis of radioactivity in half-gram samples of fat.

Table I. Residues of Radioactive Carbon in Nonfat Tissues

Tissue Analyzed[a]	GS-13005 Equivalents, P.P.M., Average
Liver	0.11
Kidney	0.04
Heart	0.02
Muscle, round	0.02
Muscle, tenderloin	0.02
Spleen	0.03
Brain	0.02

[a] Schöniger combustions.

Table II. Residues of Radioactive Carbon in Fats

Fat Analyzed[a]	GS-13005 Equivalents, P.P.M.	
Perirenal	0.03	Avg. of 4 values
Subcutaneous	0.02	Avg. of 4 values
Omental	0.03	Avg. of 2 values
Tail head[b]	0.02	One value, very little sample

[a] Solubilization in base (NCS) and subsequent addition of toluene scintillation liquid.
[b] Fat taken from the tail where it is attached to the rump.

The data for all tissues indicate that, for a cow fed this pesticide at the rate of 1 mg. per kg. per day over a five-day period, no significant build-up of GS-13005 or its metabolites occurred—i.e., the amount of radioactivity found in each tissue, with the exception of the liver and kidney, was only two- or threefold more than 0.01 p.p.m. equivalent to GS-13005, the limit of detection in this study. Even the values for the liver and kidney were low, 0.11 and 0.04 p.p.m., respectively.

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