Table III. Guthion Metabolite Residues[a] in Milk during and after Feeding of Insecticide to Dairy Cattle

	Level Fed					
	8.3 P.P.M.		16.7 P.P.M.		33.3 P.P.M.	
	Animal No.					
Days on Feed	528	540	928	981	531	532
0	<0.02	<0.02	0.03	0.08	0.15	0.17
1	0.04	<0.02	0.07	0.08	0.18	0.26
2	0.03	0.05	0.10	0.12	0.30	0.24
3	0.05	0.04	0.20	0.29	0.13	0.19
4	0.03	0.05	0.13	0.14	0.12	0.21
5	0.03	0.03	<0.02	0.10	0.19	0.28
6	0.05	0.03	<0.02	<0.02	0.14	0.15
7	<0.02	<0.02	0.05	0.03	0.07	0.07
10	0.03	<0.02	0.07	0.09	0.05	0.06
14	<0.02	0.06	<0.02	0.02	0.21	0.14
Av. value[b]	0.030		0.076		0.165	
Days Posttreatment						
3	<0.02	...	<0.02	<0.02	<0.02	<0.02
7	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
10	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02

[a] Net residue values expressed as p.p.m. mercaptomethylbenzazimide.
[b] In calculating averages, values recorded as <0.02 p.p.m. assumed to be 0.02 p.p.m.

buffer solution were added. The buffer consisted of 15 parts of 0.2*M* disodium phosphate and 85 parts of 0.1*M* citric acid in isopropyl alcohol–water (1 to 1). Fluorescence measurements were made as previously described (*1*), except that the activating and fluorescence wavelengths were 330 and 425 mμ, respectively, and the instrument was not standardized with a quinine sulfate solution.

A standard curve established that a linear response is obtained for the range of 0 to 30 μg. per 8 ml. (final volume), or 0 to 0.75 p.p.m. based on a 200-gram sample. The internal standard is used to compensate for any quenching of the fluorescence resulting from the presence of the milk extract.

Recovery experiments were conducted using Guthion and its oxygen analog. Samples were spiked in the initial blending step.

The results in Table I demonstrate that the method is satisfactory for the two compounds.

On the basis that samples must have a net fluorescence equal to or greater than the untreated control in order to contain a reportable residue, the sensitivity of the method for Guthion is approximately 0.02 p.p.m. and for the oxygen analog and other benzazimide-containing moieties is 0.03 p.p.m.

Results and Discussions

No detectable residues of Guthion were found in any of the milk samples, regardless of feeding rate. However, the results for the chloroform-acetone eluate (Tables II and III) indicate that significant residues of the oxygen analog of Guthion and/or other benzazimide-containing moieties were present at all feeding levels. These residues appeared within 1 day after the treatment was started and disappeared within 3 days after treatment was discontinued. The mean values show that there is approximately a straight-line relationship between the amount fed and the metabolite level in the milk.

For reasons discussed previously (*2*), the results in Tables II and III are expressed as residues of mercaptomethylbenzazimide, corrected for fluorescence yield. The control value of 0.026 p.p.m. of Guthion in Table I when expressed on the same basis is 0.021 p.p.m. This accounts for the stated sensitivity of 0.02 p.p.m. in Tables II and III.

Acknowledgment

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RESIDUES

Nature and Extent of Guthion Residues in Milk and Tissues Resulting from Treated Forage

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THE usefulness of Guthion (*O,O*-dimethyl *S* - 4 - oxo - 1,2,3 - benzotriazin-3(4*H*)-ylmethyl phosphorodithioate) in controlling a variety of insects on forage crops made it necessary to determine the nature and extent of milk residues that might result from feeding forage contaminated with Guthion residues.

Previous work (*2*) has shown that feeding rates equivalent to 4.2 to 33.3 p.p.m. in fresh forage do not result in detectable residues of Guthion in milk but do result in significant residues of one or more metabolites.

To learn more regarding the nature of these metabolites additional studies were conducted using P^{32}- and C^{14}-labeled Guthion. Furthermore, since at this point it was well established that proposed label recommendations (one application of 12 ounces active per acre, 21-day preharvest interval) could result in residues in the 1-p.p.m. range in fresh alfalfa, it was considered desirable to carry out an additional low-level feeding test at this level.

Experimental Methods

Studies with Radiolabeled Guthion. PHOSPHORUS-LABELED MATERIAL. Guthion-P^{32} was obtained from Farbenfabriken Bayer A.G., Leverkusen, Germany. An oral dose of 5 mg. per kg. of the radioactive compound (specific activity, 1.23 mc. per gram) was administered to a 364-kg. cow. This was calculated to be equivalent to 69.5 p.p.m. in one day's feed (*2*). The animal was kept in a metabolism stall for 5 days. Milk and blood samples were collected at regular intervals. Urine and feces samples were collected at time of elimination and the time of collection was recorded. On the fifth day the animal was sacrificed and various tissue samples were collected. All samples were kept frozen until they could be analyzed.

Treatment of alfalfa with Guthion according to label recommendations will usually result in fresh forage residues of 1 p.p.m. or less. Very occasionally residues in the 1.0- to 1.5-p.p.m. range may be encountered. Use of such forage will not result in detectable residues in milk of either Guthion or its oxygen analog or residues greater than 0.01 p.p.m. of phosphorus-free metabolites containing the benzazimide structure. The exact nature of the metabolites has not been determined.

All measurements were made with a Nucleonic Corp. of America Model M4 jacketed dip counter tube. Total radioactivity in milk and urine was determined directly. Blood samples were also counted directly after dilution with 1.5 volumes of physiological saline solution. Organosolubles in blood were measured by extracting the diluted blood with an equal volume of chloroform and counting aliquots of the chloroform phase. Total radioactivity in feces and tissues was determined by digesting 10 grams of the material with 100 ml. of a mixture containing 50 ml. of concentrated sulfuric acid, 70 ml. of 70% perchloric acid, and 880 ml. of concentrated nitric acid per liter. More nitric acid was added when necessary to complete the digestion. Digests were diluted to a definite volume and counted.

Tissue and milk samples were extracted essentially as described in the residue procedure (*1*). The acetonitrile extracts obtained were combined and evaporated to dryness and the resulting residue was dissolved in chloroform for counting. Recoveries of Guthion-P^{32} and its oxygen analog from brain, fat, heart, kidney, liver, steak, and milk ranged from 87 to 100%. Urine samples were examined for metabolites using the ion exchange procedure described by Plapp and Casida (*4*).

Carbon-Labeled Material. Synthesis. Radioactive Guthion was prepared by the following series of reactions. The compound was labeled at the methylene carbon group.

Benzazimide (0.90 gram, 100% purity), radioactive paraformaldehyde (0.272 mc., 0.0209 gram), and 0.173 gram of 95% paraformaldehyde were placed in a three-necked 50-ml. flask with 15 ml. of ethylene dichloride. The slurry was heated to 40° C. with stirring and 0.9 gram of thionyl chloride was added through a dropping funnel while the temperature was maintained at 40° C. The temperature was then raised slowly to 65° C. and held there until gas evolution ceased. After cooling to 30° C., 4.1 ml. of water were added and the mixture was neutralized with 50% sodium hydroxide to pH 6. After filtering and washing, the solvent was evaporated under reduced pressure at room temperature. The weight yield of chloromethylbenzazimide was 97.5% of theoretical. The product was placed in a 50-ml. three-necked flask together with 3.8 ml. of ethylene dichloride. After heating to 50° C. with stirring, 1.81 ml. of a solution of 0.05 gram per ml. of sodium bicarbonate and 4.0 grams of a 36.0% solution of *O,O*-dimethyl phosphorodithioate sodium salt in water were added. The mixture was held at 60° to 65° C. for 4 hours. After cooling to 30° C., 5 ml. of ethylene dichloride was added and the mixture was poured into a separatory funnel and shaken. Following re-extraction of the aqueous phase with ethylene dichloride, the solvent was evaporated under reduced pressure at room temperature. A crude yield of 1.718 grams of Guthion was obtained. The crude product was purified by recrystallizing twice from methanol, followed by chromatography on a 30-gram column of Florisil containing 1.25% water. The column was prepared by slurrying the Florisil in 10% (v./v.) benzene-chloroform. The Guthion was applied in a small volume of the same solvent and eluted with 300 ml. of additional solvent.

Chromatography on formamide-treated paper using toluene-isooctane (1 to 1 v./v.) indicated that the product was radiochemically pure. The formamide-treated paper was prepared by passing S & S 2040b paper through a 25 to 75 (w./v.) mixture of formamide and acetone. The over-all yield of Guthion-C^{14} was 60.6%.

Animal Treatment. Guthion-C^{14} was administered orally at the rate of 2.66 mg. per kg. to a 364-kg. cow. This dose was equivalent to 37 p.p.m. in green forage. The animal was kept in a metabolism stall for a 3-day period following treatment. During this time milk samples were collected at the usual milking times. Samples were kept frozen until they could be analyzed.

Examination of Milk Samples. C^{14} activity was measured using a Packard Tri-Carb liquid scintillation spectrometer. Whole milk samples were counted using a method similar to that described by Steinberg (*5*). Six milliliters of milk were pipetted into 2 grams of anthracene along with 100 μl. of Triton X-100. The samples were counted in the usual manner and corrected for counting efficiency using sodium carbonate-C^{14} as an internal standard. A toluene-based liquid scintillator solution was used for the other samples. Benzoic acid-C^{14} was used as internal standard. Lactose derivatives were suspended on Cab-O-Sil before addition of the liquid scintillation solution.

Milk samples were processed as described in the residue method (*1*). The milk fractionation scheme is shown in Figure 1. At various intermediate stages in the procedure aliquots of the sample were taken for counting. The items underlined in the flowsheet in Figure 1 indicate the points when samples were taken. The aqueous acetone and Skellysolve B fractions are discarded in the residue procedure (*1*). Ultimately, portions of some of the samples were analyzed fluorometrically and radiometrically so that the two procedures could be compared. Recoveries of total radioactivity from the alumina column (see Figure 1) were approximately 95%.

Acetonitrile extracts (Figure 1) were chromatographed on formamide-treated paper prepared as previously described. Following development periods ranging from 3 to 24 hours, the paper chromatograms were either cut into 1-cm. lengths for liquid scintillation counting, or sprayed to develop a color. In the latter case, drops of 0.04% methyl red solution were spotted on opposite corners of the chromatogram, which was then sprayed lightly with a mixture containing 80 ml. of 0.5*N* KOH in isopropyl alcohol, 10 ml. of propylene glycol, and 10 ml. of distilled water. The chromatograms were then placed in an 85° C. oven for 2 minutes. The procedure was repeated twice, at which point the paper was sprayed with 3*N* hydrochloric acid until the indicator spots appeared red. Then the chromatogram was sprayed with a 0.25% (w./v.) sodium nitrite solution. After a 10-minute wait a 2.5% ammonium sulfamate spray was applied. Finally, after a second 10-minute waiting period the chromatogram was sprayed with a 1% (w./v.) solution of *N*-(1-naphthyl)ethylenediamine dihydrochloride solution. The paper was kept moist by spraying periodically with the dye reagent for 10 minutes and then with a 20% isopropyl alcohol–80% water solution for 50 minutes.

Low-Level Feeding Studies with Nonradioactive Guthion. Eight Holstein cows weighing from 360 to 460 kg. were used in this test. Feeding rates were 0.05, 0.10, and 0.20 mg. of Guthion per kg. of body weight per day. Based on the considerations previously outlined (*2*) these rates are equivalent to 0.7, 1.4, and 2.8 p.p.m. of Guthion in fresh forage. Two animals served as controls. Milk samples were collected during a 17-day treatment period and, in the case of the 2.8-p.p.m. level, for 3 days after treatment. The two 1.4-p.p.m. animals were slaughtered on the last day of treatment and various tissue samples were collected. The other animals were not sacrificed. The fluorometric method involving use of the isoelectric cleanup procedure (*1*) was used for analysis of all samples.

Results and Discussion

Studies with Phosphorus-Labeled Guthion. The radioactivity in blood is plotted against time in Figure 2. A small radioactive peak appeared 15 minutes after treatment. Since this activity dropped to a negligible value at

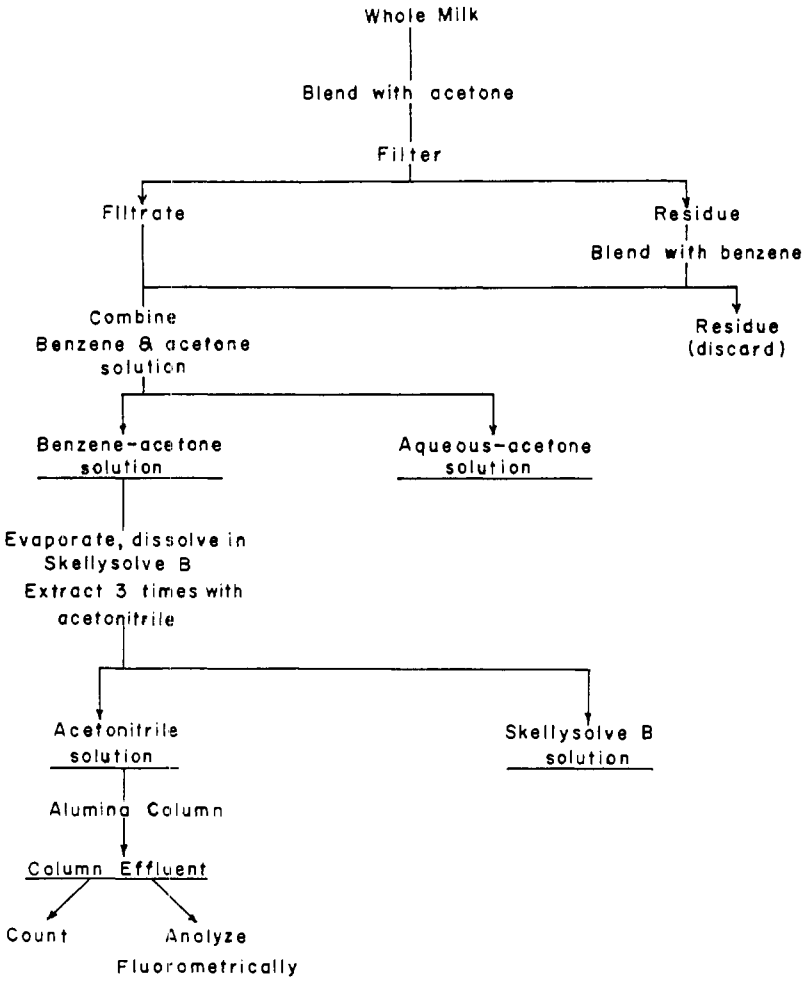


Figure 1. Milk fractionation scheme

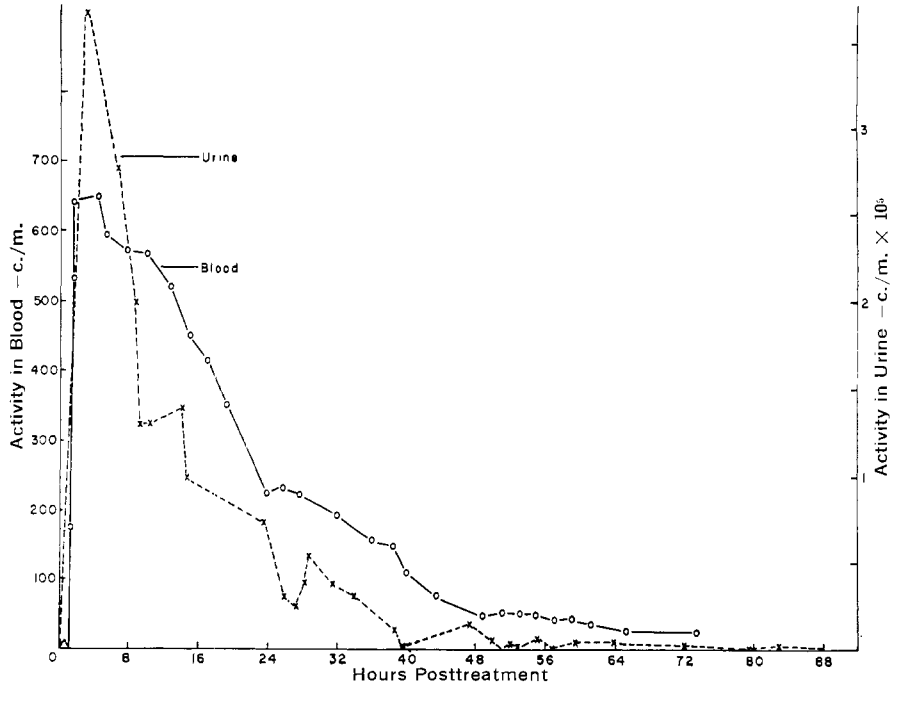


Figure 2. Total activity in blood and urine following oral administration of Guthion-P^{32}

the 30-minute sampling, it may have been due to an impurity in the Guthion that was very rapidly absorbed. One hour after treatment the activity in the blood rose sharply. A maximum was reached between 4 and 6 hours, followed by a sharp decline for the next 30 hours. The amount of activity in the blood declined gradually thereafter. No chloroform-soluble activity was found when samples containing the highest activity were partitioned against chloroform. This indicates that Guthion or its oxygen analog is not present in blood and therefore they are either not absorbed into the blood or are very rapidly hydrolyzed.

The pattern of elimination of phosphorus-32 in urine is also shown in Figure 2. The changes in activity closely parallel those found in the blood. When urine samples were chromatographed on an ion exchange column according to the procedure of Plapp and Casida (*4*), at least six and possibly eight peaks were observed (see Figure 3). Most of the activity falls in peak I, at first thought to be phosphoric acid. However, when this material was analyzed directly by the phosphomolybdate method of Martin and Doty (*3*), no more than 10% of the activity partitioned into the benzene phase. If the material in peak I had been phosphoric acid, the extraction into benzene would have been quantitative. No attempt was made to identify the other hydrolysis products.

The per cent of administered dose excreted after various time intervals is shown in Figure 4. No appreciable activity was observed in the feces during the first 4 hours. The activity then increased continuously during the next 30 hours. Forty per cent of the administered dose was excreted in the urine; 17% in the feces. The remainder of the administered material presumably entered the phosphorus pool.

The radioactivity levels in milk at various times are shown in Figure 5. The activity rose rapidly during the first 24 hours, remained relatively high through 48 hours, then gradually de-

Table I. Residues of Radioactive Phosphorus in Tissues

Tissue	Guthion Equivalents, P.P.M. Total	Organo-soluble
Brain	0.01	<0.005
Fat (omental)	0.56	<0.005
Fat (renal)	<0.005	<0.005
Fat (subcutaneous)	0.08	<0.005
Heart	0.12	0.01
Kidney	0.57	0.06
Liver	1.46	0.10
Meat, brisket	0.04	<0.005
Meat, round steak	...	<0.005
Meat, sirloin steak	0.04	<0.005
Udder	0.14	0.02

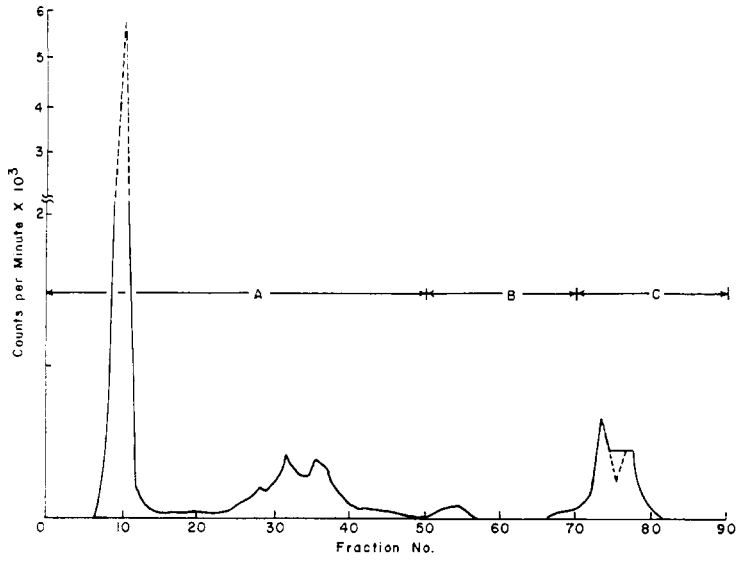


Figure 3. Ion exchange fraction of P^{32} activity in urine

Elution solvents
A. HCl, pH 2 to HCl, pH 1
B. HCl, pH 1 : methanol : : 1 : 3 to HCl, 1N : methanol : : 1 : 3
C. HCl, 1N : methanol : : 1 : 3 to HCl : water : methanol : : 1 : 1 : 6

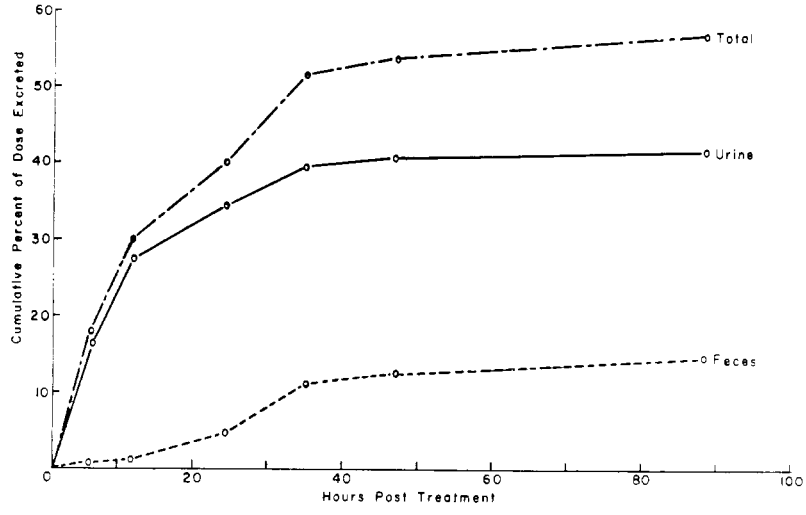


Figure 4. Cumulative per cent of P^{32} excreted in urine and feces

clined. Maximum activity was found in the 24-hour sample. However, when this sample, containing the equivalent of 2.16 p.p.m. of Guthion, was carried through the extraction procedure as described in the experimental section, no more than 0.003 p.p.m. was present in the form of Guthion or its oxygen analog.

Total and organosoluble activity in the various tissues was determined by the combustion and extraction procedures previously described. Tissues which had the highest organosoluble residues were kidney (0.06 p.p.m.) and liver (0.10 p.p.m.)

Studies with Carbon-Labeled Guthion. The results of the P^{32} study clearly indicated that Guthion residues in milk are actually due to phosphorus-free metabolites containing the benzazimide structure. However, questions remained as to the number and nature of the metabolites. Furthermore, a possibility existed that some of the metabolites might not survive the various extractions and cleanup steps employed in the residue method. Also, there was no assurance that the yield of anthranilic acid would be the same for the metabolites as for Guthion and its oxygen analog. Justification for labeling the methylene group is based on the observation that only benzazimide derivatives substituted in the 3 position yield anthranilic acid under the conditions of hydrolysis used in the residue method. Benzazimide and hydroxymethylbenzazimide were ruled out as possible metabolites when it was shown that apparent residues did not increase when acetonitrile extracts of treated samples were refluxed with alkali. Both of the compounds will survive the extraction and cleanup steps and be hydrolyzed to anthranilic acid when refluxed with alkali.

Table II. Fluorometric and Radiochemical Methods on Acetonitrile Fraction[a]

Sample Time, Hr.	Radiochemical, P.P.M.	Fluorometric, P.P.M.	Ratio of Values
8	0.086	0.082	0.953
20	0.115	0.091	0.791
32	0.101	0.074	0.733
44	0.069	0.054	0.783
56	0.050	0.043	0.860
68	0.031	0.020	0.645

[a] Results reported as p.p.m. of mercaptomethylbenzazimide.

The activity in whole milk at various intervals after treatment is plotted in Figure 6. Slightly less than 1% of the total dose was excreted in milk during a 3-day posttreatment period.

The ratio of radioactivity in the aqueous-acetone phase and the benzene-acetone fraction (see Figure 1) is plotted against time in Figure 7. Excluding the initial sampling time, approximately 40% of the radioactivity in the milk was found in the benzene-acetone phase. The data show that in 24 hours a steady state is reached. The proportion of the activity in the organosoluble fraction which partitioned between acetonitrile and Skellysolve B is plotted in Figure 8. It is evident that the proportion of acetonitrile-extractable material decreases from almost 100% at 8 hours to about 80% in 70 hours. This may mean that with increasing time more and more activity is incorporated into naturally occurring fatty substances.

Portions of the chromatographed acetonitrile fraction were analyzed both by the photofluorometric method (*1*) and radiochemically. Based on the fact that each molecule of Guthion contained one methylene group and forms one molecule of anthranilic acid, the results should be directly comparable if the ratio of methylene to benzazimide groups is the same in the residue as in the original compound.

The results in Table II indicate that, aside from the 8-hour sample, the fluorometric method gives, on the average, 76.2% of the value obtained by the radiochemical method. Evidently the yield of anthranilic acid from the metabolites is not quantitative or there is a certain amount of completely metabolized radiocarbon in the acetonitrile fraction. As the acetonitrile fraction contains phospholipids, the latter is probable.

In view of the fact that appreciable amounts of radioactivity were present in the aqueous phase, further identification studies were conducted. It was found at the outset that the entire activity of this fraction was dialyzable.

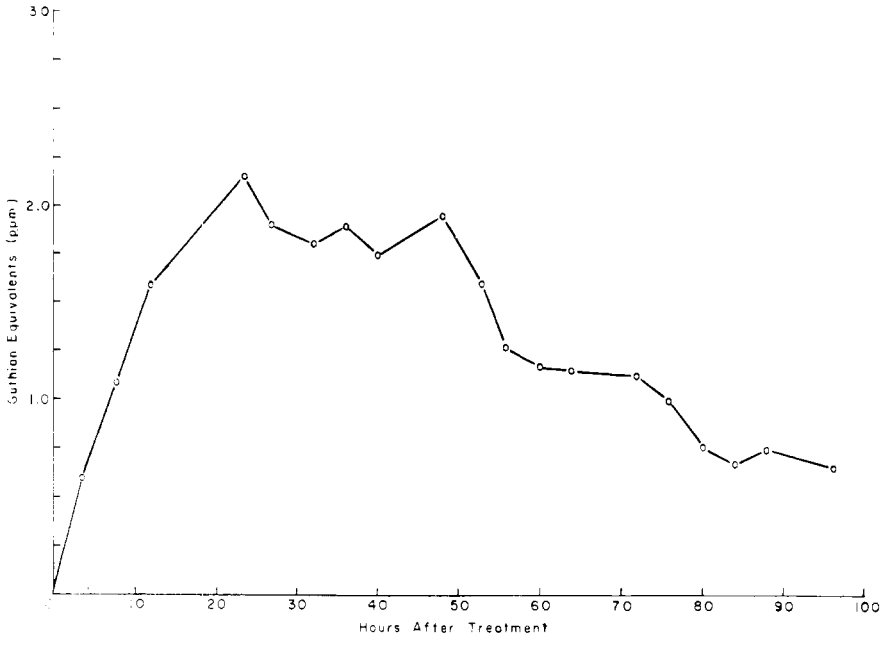


Figure 5. Total P^{32} activity in milk

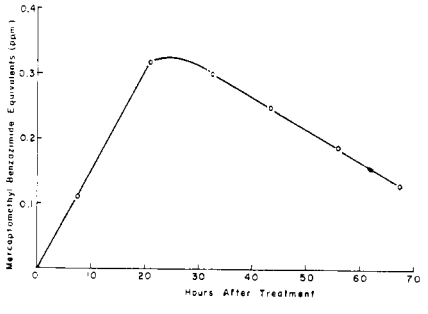


Figure 6. C^{14} metabolite residues in milk

Table III. Identification of Lactose

Derivative	Melting Point, ° C. Beilstein	Exptl.
Lactose osazone	203–215	208
Lactose acetate	86–106	90–91
Mucic acid	225	225

This indicated that the activity was present in either an ionic compound or a nonionic substance of low molecular weight. After dialysis the external liquid was concentrated and passed through a Dowex 1-X8 (chloride form) ion exchange column. All of the radioactivity passed through the resin and it was therefore concluded that the unknown metabolite was not anionic.

The effluent from the ion exchange column was passed through a column of Darco G-60. The column removed extraneous color from the solution, but all of the radioactivity passed through. The effluent was concentrated in vacuo and a thick sirup was obtained. On standing, crystallization occurred. The crystals contained radioactivity and in view of the large number obtained were suspected of being lactose. Since the activity might have been occluded in the crystals, several derivatives were prepared. Lactose osazone, lactose acetate, and mucic acid were prepared. Melting points were determined on the preparations (Table III).

It was impossible to count the osazone because of color quenching, but the lactose acetate and mucic acid were easily counted. The results showed that two thirds of the activity was in the glucose and one third in the galactose portion of the molecule. The total activity in lactose accounted for 68% of the radioactivity in the aqueous fraction. These data show that the methylene group of Guthion is metabolized and incorporated into sugars. It is entirely possible that the portion of water-soluble activity not accounted for was also in the form of naturally occurring substances.

The acetonitrile fraction (Figure 1) apparently contains compounds containing the benzazimide structure. Evaporation of the acetonitrile extract followed by partitioning between chloroform and acid or base did not result in any loss activity.

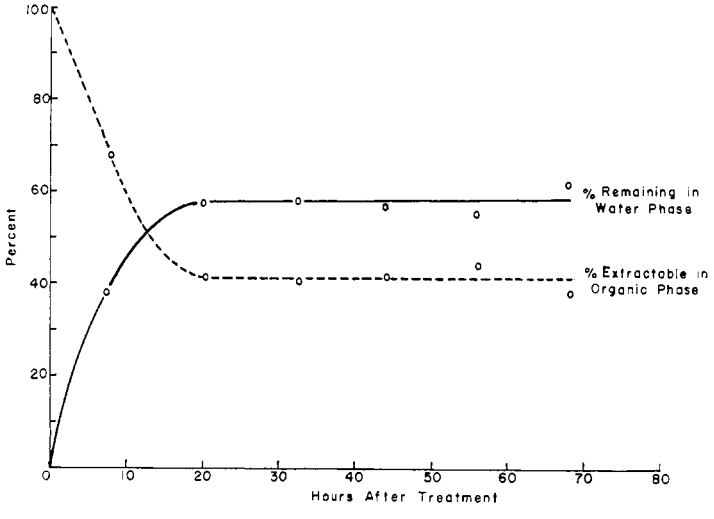


Figure 7. Distribution of C^{14} activity following extraction

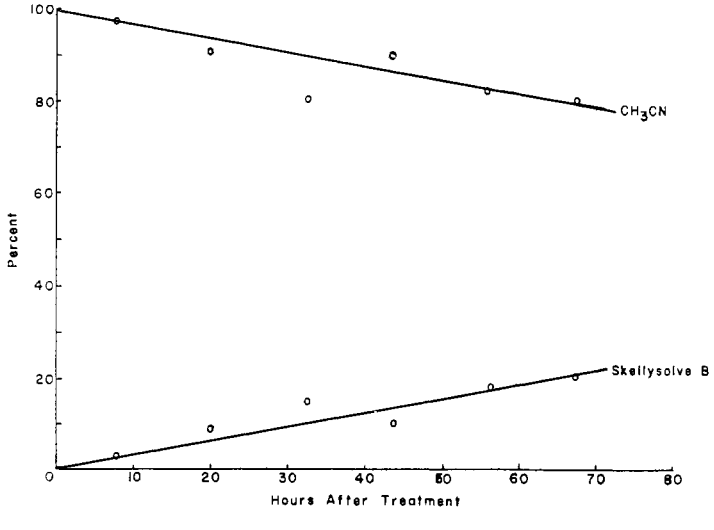
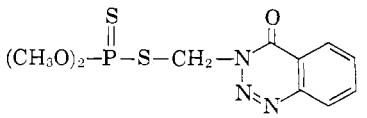
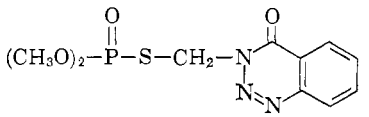
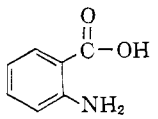
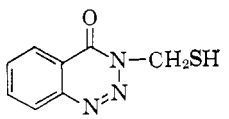
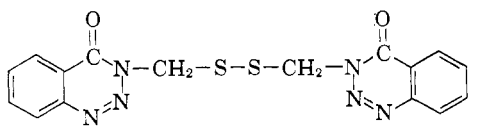
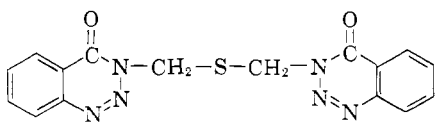


Figure 8. Amount of organic extractables partitioning into acetonitrile and Skellysolve B

Table IV. R_f Values for Guthion and Various Possible Metabolites

Compound	Structure	R_f
Guthion		0.86
Guthion oxygen analog		0.24
Anthranilic acid		0.02
Mercaptomethyl-benzazimide		0.69
Methyl benzazimide disulfide		0.00
Methyl benzazimide sulfide		0.45
Unknown		0.00–0.06

This fraction was chromatographed on formamide-treated papers together with the various compounds listed in Table IV. After a 3-hour development period all of the activity applied remained at or very near the origin, whereas many of the possible metabolites had moved a considerable distance (Table IV). After a 24-hour development period during which the solvent front moved off the paper, the unknown activity was separated into four distinct spots. The areas of radioactivity coincided exactly with the colored spots produced when the spray procedure was used. In the case of the 24-hour development period, all of the knowns in Table IV, with the exception of anthranilic acid and methyl benzazimide disulfide, had moved off the paper. None of the activity of the unknown(s) moved off the paper. Anthranilic acid and methyl benzazimide disulfide were not separated from the unknown spots. Anthranilic acid can, of course, be ruled out as a possible metabolite because all of the unknowns were radioactive and anthranilic acid could not have been radioactive because of the type of label used. Methyl benzazimide disulfide corresponds exactly with the unknown component of lowest mobility but it is probably not a metabolite because omitting the alkali spray treatment eliminated color development of the compound but not of the unknown.

The relative amount of activity in the four spots at various time intervals after treatment is given in Table V.

The proportion of the various metabolites remained relatively constant over the interval studied. A very high percentage of the activity was in one spot.

Low-Level Feeding Study with Nonradioactive Guthion. The milk residue data for the 0.7-, 1.4-, and 2.8-p.p.m. feeding rates, presented in Table VI, are expressed as residues of mercaptomethylbenzazimide. This was done because mercaptomethylbenzazimide has the highest possible molecular weight of any phosphorus-free benzazimide derivative containing both the benzazimide and methylene moieties. Therefore, the results shown in Table VI are maximum. Residues were corrected for the 76.2% recovery of anthranilic acid obtained from the metabolites. Since the molecular weights of mercaptomethylbenzazimide and Guthion are 193 and 317, respectively, the original results, expressed as Guthion equivalents, were multiplied by (193)/(317)(0.762) to obtain the results in Table VI.

The feeding study confirmed the results found previously (*2*), that the amount of the metabolite in milk reaches a constant value in 24 hours. The results shown in Table VI are means of results obtained from the first to the seventeenth day of feeding.

Table VI shows that a nearly direct relationship exists between the amount of residue found and the feeding rate. The reproducibility of the results is remarkable considering the low levels of the residues being determined. The net residue reported for the 0.7-p.p.m. feeding rate is probably not significant. It is evident from these results that feeding Guthion at the rate of 2 p.p.m. in green fodder will produce a residue of Guthion metabolites of about 0.008 p.p.m. in milk. Thus, use of Guthion according to label recommendations will not result in

Table V. Relative Distribution of Unknown Radioactive Metabolites in Milk at Various Times after Treatment

Unknown	Distance Moved,[a] Cm.	Per Cent 8 hr.	32 hr.	68 hr.
1	0.0	3.0	3.3	1.1
2	4.0	8.7	8.6	5.5
3	12.5	...	2.6	3.1
4	22.0	88.3	85.5	90.3

[a] R_f values could not be calculated because it was necessary to allow solvent to run off the paper in order to get a good separation.

Table VI. Total Guthion Metabolite Residues in Milk

Feeding Rate, P.P.M.	Gross Residue	Net Residue[a,b]
0	0.004 ± 0.001	
0.7	0.006 ± 0.002	0.002 ± 0.002
1.4	0.009 ± 0.002	0.005 ± 0.002
2.8	0.017 ± 0.003	0.012 ± 0.003

[a] Calculated as mercaptomethylbenzazimide, corrected for 76.2% recovery of anthranilic acid from metabolites.

[b] Mean values followed by average deviation for milk samples taken during 17-day treatment period. Samples taken daily for first 10 days and then on 13th 15th, and 17th days.

Table VII. Total Metabolite Residues in Tissues of Cows on 1.4-P.P.M. Guthion Ration

Tissue	Animal No.	Control, P.P.M.	Net Residue, P.P.M.[a]
Flank steak	1	0.02	<0.02
	4	0.02	<0.02
Round steak	1	0.02	<0.02
	4	0.02	<0.02
Brain	1	0.02	<0.02
	...	...	...
Heart	1	0.01	0.02
	4	0.01	<0.01
Liver	1	0.01	0.05
	4	0.01	<0.01
Kidney	1	0.01	0.03
	...	...	...
Fat, subcutaneous	1	0.02	0.03
	4	0.02	0.03
Fat, omental	1	0.02	0.03
	4	0.02	<0.02
Fat, renal	1	0.02	<0.02
	4	0.02	<0.02

[a] Expressed as p.p.m. of mercaptomethylbenzazimide and corrected for 76.2% recovery of anthranilic acid from metabolites.

residues in excess of 0.01 p.p.m. of metabolites in the milk of cows fed treated forage.

A study of the cows on the 2.8-p.p.m. feeding rate showed that 1 to 2 days after the feeding of Guthion ceased the residues in milk had disappeared.

Residue data for tissues from animals fed at the equivalent of 1.4 p.p.m. and sacrificed on the last day of treatment are presented in Table VII. In all cases the residues were less than 0.1 p.p.m. For ease of comparison, results for the tissues are expressed on the same basis as for milk, although no direct evidence is presented to show that the metabolites in tissue are the same as those in milk. In all probability they are.

Conclusions

The radioactive studies have established that the fluorometric method (*1*) is satisfactory for determining Guthion residues in milk and tissues. The residues observed in milk are due to four unidentified phosphorus-free metabolites which still contain the benzazimide moiety. One metabolite accounts for 85 to 90% of the residue observed. Comparison of radiochemical and fluorometric results for aliquots of the same extract indicates that the yield of anthranilic acid from the metabolites is at least 76% of theoretical. If Guthion is used according to label recommendations, the residue in the fresh forage at harvest will usually be less than 1 p.p.m. and always less than the tolerance level of 2 p.p.m. The residue in milk from feeding forage containing 2 p.p.m. of Guthion will be 0.008 p.p.m., expressed as mercaptomethylbenzazimide. Under the same conditions, tissue residues will not be in excess of 0.1 p.p.m.

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RESIDUE DETERMINATION

Spectrophotofluorometric Method for Guthion Residues in Milk and Animal Tissues

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A sensitive fluorometric method has been developed for the determination of Guthion [O,O-dimethyl *S*-4-oxo-1,2,3-benzotriazin-3(4*H*)-ylmethyl phosphorodithioate] residues in milk and animal tissues. The oxygen analog is also determined by the procedure described. The method involves an alkaline hydrolysis to give anthranilic acid which, after adjustment of the pH, is extracted into benzene and determined fluorometrically at an activating wavelength of 340 mμ and a fluorescence wavelength of 400 mμ. The sensitivity of the method is about 0.005 p.p.m. for milk, 0.02 p.p.m. for most animal tissues, and 0.03 p.p.m. for fat.

In residue analysis of crucial food products such as milk, greater sensitivity is required than for most food commodities. It was apparent that a residue method for Guthion, more sensitive than the colorimetric methods previously described (*1*, *3*), would be desirable for studies involving milk. Since previous work (*3*) has shown that Guthion is very easily hydrolyzed to anthranilic acid, a very fluorescent compound, the possibility of developing a fluorescence method suggested itself. Preliminary work indicated that the sensitivity of such a procedure would be excellent and dependent only on the degree to which the background fluorescence could be reduced.

A preliminary method was developed which gave background values for milk of about 0.03 p.p.m. (*2*). The procedure involved a column cleanup with Florisil, followed by fluorescence measurement in buffer solution. Some analyses were performed using the procedure, although it was realized that a better cleanup would eventually be required (*2*).

Consideration of the effect of hydrogen ion concentration on the fluorescence of anthranilic acid led to the hypothesis that pH 4.1 might represent the "isoelectric point" of the compound, and that at this pH it might be possible to extract anthranilic acid quantitatively from an aqueous solution with an immiscible nonpolar solvent, such as benzene, and thereby effect an additional measure of cleanup. This proved to be the case. It was found that the compound could be quantitatively extracted from a pH 4.1 buffer solution using an equal volume of benzene. There was essentially no difference in the fluorescence readings of samples buffered over the range of pH 4.0 to 4.2.

Application of the "isoelectric extraction" procedure to control milk samples indicated that the additional cleanup step, by itself, would not give control values of 0.01 p.p.m. or less. However, further work showed that a combination of chromatography on acid-washed aluminum using chloroform as solvent and isoelectric extraction would give the desired control values.

Apparatus

Chromatographic tubes, 20 × 400 mm., equipped with Ultramax stopcock and integral 300-ml. reservoir.

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