brown color on storage over time. After the formation of the Amadori rearrangement products, OVA–lac and OVA–cel complexes exhibited the same behavior in fluorescence and polymer formation and the same brown color, occurring slightly faster than in the OVA–mal system. The faster reaction of the OVA–lac and OVA–cel complexes may be attributed to the activation by a bidentate hydrogen bonding between C-3 OH and the acetal oxygen atom (Figure 7, path a). However, in the OVA–mal complex with a 1,4-linkage, unfavorable hydrogen bonding took place only between C-3 OH and the glycoside oxygen atom by steric hindrance (Figure 7, path b). For this reason, the OVA–mal system may be most stable in the formation of brown color and fluorescence. A part of the brown color formation from these OVA–disaccharide systems may have resulted in cleavage between a C-4–C-5 bond of the hydrogen-bonding intermediate with a disadvantageous conformation (Figure 8).

Registry No. Lac, 63-42-3; mal, 69-79-4; cel, 528-50-7; iso, 499-40-1; mel, 585-99-9.

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A Method for the Determination of the Main Metabolites of Malathion in Biological Samples

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A simple method has been developed for the quantification and identification of the main metabolites of malathion in biological samples. These metabolites are malathion α-monoacid, malathion diacid, *O,O*-dimethyl phosphorothioate, *O,O*-dimethyl phosphorodithioate, and desmethylmalathion. Following sorbent extraction using Bond Elut extraction columns containing anionic bonded-phase silica sorbent, the extract is methylated and quantified by use of gas–liquid chromatography equipped with a nitrogen–phosphorus selective detector. Gas–liquid chromatography—mass spectrometry is used to identify the metabolites. Recoveries of the metabolites from urine at fortification levels of 20–160 μg/mL ranged from 82 to 127% with CV <20. From feces fortified with 2–20 μg/g of the metabolites, the recoveries ranged from 18 to 66%, with CV <30.

The organophosphorus compound malathion, *O,O*-dimethyl *S*-(1,2-dicarbethoxyethyl) phosphorodithioate, is a broad-spectrum insecticide with low mammalian toxicity, which is widely used as an agricultural insecticide and as an ectoparasitic agent in both animals and humans. Malathion is activated to its oxygen analogue malaoxon, which is responsible for the toxic effects of the compound (Hayes, 1982). Degradation of malathion to inactive metabolites results from hydrolytic cleavage of one or two of the carboxyethyl ester groups, giving rise to malathion α-monoacid (MCA) or malathion diacid (DCA), respectively (Chen et al., 1969; Bradway and Shafik, 1977). Additional metabolites arise from cleavage of the P–S and S–C linkages, giving *O,O*-dimethyl phosphorothioate (DPT) and *O,O*-dimethyl phosphorodithioate (DPDT), respectively, and by demethylation, giving desmethylmalathion (DMM) (Bhagwat and Ramachandran, 1975; Nomeir and Dauterman, 1978).

A few methods for the extraction, purification, and quantification of these metabolites in different materials have been reported. However, some of these involve analysis of only the alkyl phosphate metabolites (Shafik and Enos, 1969; Shafik et al., 1971, 1973; Lores and

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Bradway, 1977). The methods currently available for the determination of both alkyl phosphate and carboxylic acid degradation products of malathion are rather time-consuming including extraction, alkylation, and several cleanup steps (Bradway and Shafik, 1977; EPA, 1977), or they involve determination of the metabolites in nonbiological materials (Abdel-Kader and Webster, 1981). In this paper we present a method developed for the rapid determination of the most abundant metabolites of malathion in mammals. The method involves sorbent extraction by use of commercial available trimethylaminopropyl-bonded silica cartridges, derivatization, liquid–liquid extraction, gas chromatographic quantification, and mass spectrometric identification.

EXPERIMENTAL SECTION

Chemicals. Standards of malathion monocarboxylic acid, malathiondicarboxylic acid, *O,O*-dimethyl phosphorothioate, *O,O*-dimethyl phosphorodithioate, and desmethylmalathion were gifts from American Cyanamid Co. (Princeton, NJ). Commercially available Bond Elut (Sax) cartridges containing anionic trimethylaminopropyl-bonded silica were obtained from Analytichem International (Harbor City, CA). A Vac Elut multiple sample processing station (Analytichem International, Harbor City, CA) was used for the simultaneous process of up to 10 Bond Elut cartridges. *N*-Methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald) was purchased from Aldrich Chemie (Steinheim, Federal Republic of Germany). Glusulase, containing 111 000 Fishman units of β-glucuronidase and 14 600 Fishman units of sulfatase, was obtained from E. I. du Pont de Nemours & Co. (Boston, MA). Acetone, methanol, and cyclohexane (Fisons plc, Loughborough, England) were all of high-performance liquid chromatographic quality grade.

Diazomethane reagent was generated from the action of alkali, 2 g of potassium hydroxide dissolved in 20 mL of methanol, on 8 g of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide in the presence of 100 mL of ether and codistillation of the product using a hot water bath. The diazomethane reagent was stored in a freezer until use.

Safety Precautions. Diazomethane presents explosion and toxicity hazards, and proper precautions should be taken when the reagent is generated and handled.

Biological Samples. The biological samples used during the development of the method were urine and feces collected from several adult ewes, 1 year of age, weighing about 65 kg. The ewes had not been exposed to any drugs or pesticides prior to sample collection.

Procedure. *Extraction and Derivatization.* One milliliter of biological sample, consisting either of 100 μL of urine diluted to 1 mL with distilled water or of 1 mL of feces extract prepared by homogenizing 5 g of feces with 25 mL of methanol, was carried through the following procedure. The samples were alkalized to pH 8 with 2 N NaOH and submitted to sorbent extraction.

One Bond Elut Sax column for each sample was inserted into the Vac Elut, which was attached to a vacuum source. Each column was prewashed with one column volume of methanol to solvate the sorbent and then rinsed with one column volume of distilled water. The sample was added and the vacuum reapplied to draw the sample matrix through the column, whereas the metabolites were retained on the sorbent. After the sorbent was washed with two column volumes of distilled water, the metabolites were eluted from the sorbent into 2-mL collecting vials by addition of 1 mL of 4 M HCl in methanol to each column. The methanol extracts were transferred to test tubes, two drops of 2 M HCl and 1 mL of acetone were added, and the resultant mixture was allowed to stand for 1 h. Thereafter, the samples were methylated by dropwise addition of the ethereal solution of diazomethane until a yellow color persisted in the mixture. After about 1 min, the excess diazomethane was removed by evaporation using N_2 gas. A 2-mL portion of acetone and 5 mL of cyclohexane were then added, and the sample was mixed for 30 s on a whirl mixer. The organic layer was separated and the final volume adjusted to 10 mL with cyclohexane.

To release glucuronide- and sulfate-conjugated metabolites that may be produced during the biological transformation of malathion, parallels of 1 mL of the biological samples were acidified to pH 5.2 with 20% glacial acetic acid and hydrolyzed by addition of 50 μL of Glusulase. Incubation of the samples lasted for 4 h at 37.5 °C. The samples were then treated as described above.

Quantification and Identification. Aliquots of 2 μL of the cyclohexane extract were analyzed by gas–liquid chromatography (GLC) using a nitrogen–phosphorus-sensitive detector (NPD) in phosphorus mode. The identities of the metabolites were confirmed by examining volumes of 1 μL of the cyclohexane extracts by GLC–mass spectrometry (GLC–MS), using the peak finder and library search programs. In the library, mass spectra of the metabolite standards had been included. Because of 10^{-2}–10^{-3} lower sensitivity of the GLC–MS used in peak finder mode compared to the GLC–NPD, mass spectrometric confirmation of the metabolites at the lower concentration range was done by use of selected-ion monitoring (SIM), where three to five of the most prominent ions of the mass spectra were selected.

Preparation of Standard Solutions. Stock solutions of the metabolite standards were individually prepared in acetone, and working standards of the metabolites were individually prepared from the stock solutions at concentrations ranging from 1 μg/mL to 2 mg/mL. One-hundred-microliter portions of the working standards of the individual metabolites were mixed and methylated to give standard solutions of different concentrations to use for quantitative purposes.

Analytical Quality Assurance (AQA). Experiments were carried out to determine the degrees of recoveries of the metabolites in the extraction procedure. Known amounts of the working standards were added to normal samples of urine and feces at five concentration levels to cover the concentration range of 20–160 μg/mL of urine and 2–20 μg/g of feces. Two to six parallels of each concentration were carried through the procedure. Recoveries of the metabolites from spiked biological samples were calculated from the chromatographic responses by reference to pure mixed methylated standards of equivalent concentration. The unprocessed methylated standards were assigned the value of 100%. For quantification of DPT and DMM, each giving rise to two peaks after methylation, the heights of the sum of the two peaks were used.

To further evaluate repeatability, 12 samples of normal urine or feces spiked with 80 μg/mL or 20 μg/g, respectively, of the metabolites were carried through the procedure.

Calibration curves for the quantification of the metabolites were prepared by adding the metabolites to normal samples of urine and feces to give final concentrations of 2–267 μg/mL of urine or 2–20 μg/g of feces. These spiked samples were taken through the assay procedure, and a calibration curve was constructed for each metabolite by plotting metabolite concentration versus peak height.

Instrumentation. A Carlo Erba HRGC 5300 gas chromatograph (Carlo Erba Strumentazione, Milano, Italy) equipped with a nitrogen–phosphorus-sensitive flame ionization detector (NPD) and a DB-1 fused silica capillary column (25 m × 0.32 mm (i.d.)) (J&M Scientific, Folsom, CA) was used. The injector and detector temperatures were 260 and 270 °C, respectively, and the column temperature was programmed from 60 to 100 °C at 40 °C/min, from 100 to 140 °C at 10 °C/min, from 140 to 240 °C at 40 °C/min, and for 5 min isothermal at 240 °C. The carrier gas was He at 3 mL/min, and the flow rates of hydrogen and air were adjusted for optimal conditions, approximately 30 and 350 mL/min, respectively. The splitless injector inlet purge delay time was 30 s. The peak heights and elution times were measured by a CI-10 integrator (L.D.C. Milton Roy, Shannon, Ireland).

For confirmation of the identities of the metabolites, a Hewlett-Packard 5992 GLC–MS system equipped with a 25 m × 0.32 mm (i.d.), SE-30 fused silica capillary column was used. The operation temperatures were as follows: column, 100–300 °C programmed at 16 °C/min; injector, 50 °C (on-column injection); ion source, 230–260 °C. Helium at a flow rate of 2 mL/min was used as the carrier gas. The electron energy was 80 eV.

RESULTS AND DISCUSSION

The selectivity of the assay was confirmed by taking normal urine and feces through the sample cleanup procedure. No endogenous interference was encountered for these samples, even when hydrolyzed with Glusulase.

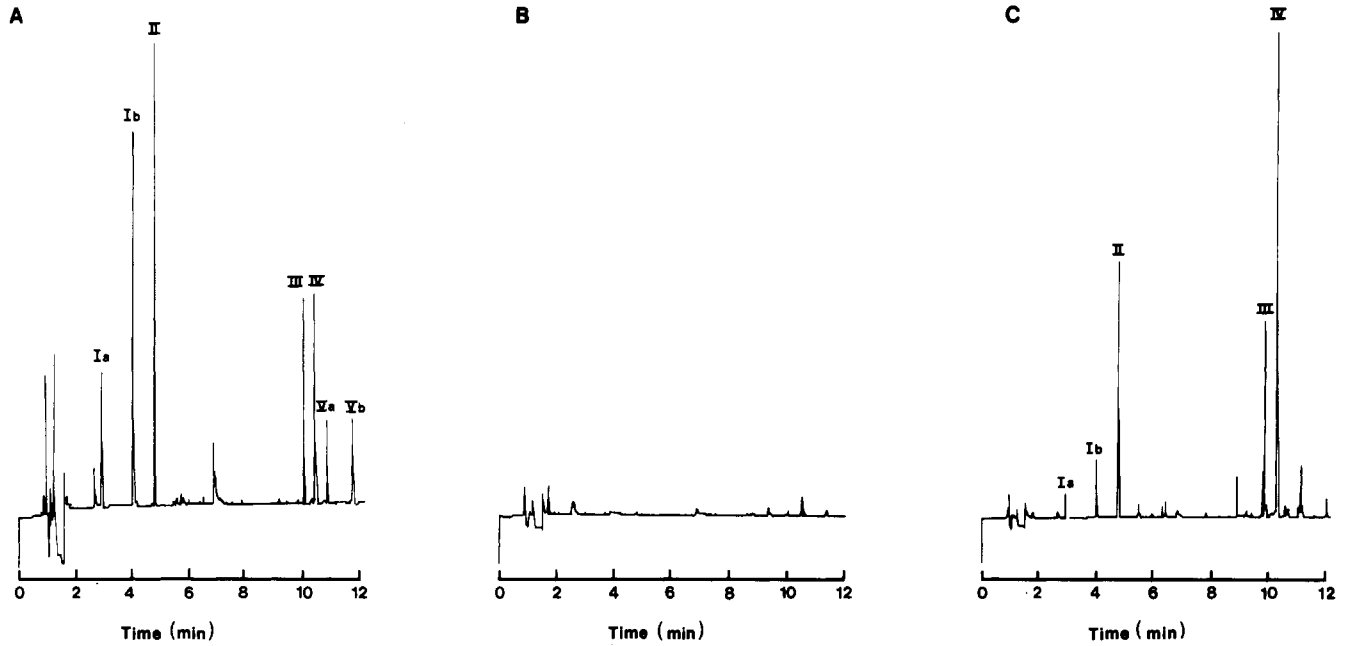

Figure 1. Gas chromatograms: (A) 2 μL of a 1 μg/mL cyclohexane solution of the mixed methylated standards of the metabolites; (B) 2 μL of a cyclohexane extract of a methylated normal urine sample; (C) 2 μL of a cyclohexane extract of a methylated urine sample collected 2 h after administration of 10 mg/kg of malathion to sheep. Gas chromatography was carried out on a DB-1 fused silica capillary column using a nitrogen–phosphorus-sensitive detector. The column temperature was programmed from 60 to 100 °C at 40 °C/min, from 100 to 140 °C at 10 °C/min, from 140 to 240 °C at 40 °C/min, and for 5 min isothermal at 240 °C. The peak numbers correspond to the numbers in Table I.

Table I. Fragmentation Pattern of the Methylated Main Metabolites of Malathion[a]

compd	chemical name	ret time, min	M^+: prominent frag, *m/e* (rel abund)					
I	*O,O*-dimethyl phosphorothioate	2.2	156 (65)	126 (43)	125 (15)	93 (100)	79 (17)	63 (26)
		2.6	156 (63)	126 (29)	110 (100)	109 (62)	80 (23)	79 (77)
II	*O,O*-dimethyl phosphorodithioate	3.3	172 (100)	125 (55)	109 (13)	93 (59)	79 (22)	63 (27)
III	malathion diacid	6.3		158 (62)	145 (100)	125 (91)	113 (67)	93 (74)
IV	malathion α-monoacid	6.4		159 (100)	158 (51)	125 (81)	113 (43)	99 (14)
V	desmethylmalathion	6.6		173 (100)	158 (34)	127 (81)	125 (67)	93 (56)
		7.3		173 (34)	127 (100)	125 (23)	99 (48)	79 (21)

[a] Amount injected was 100 ng. GLC retention times on a SE-30 fused silica capillary column (25 m × 0.32 mm (i.d.)) programmed from 100 to 300 °C at 16 °C/min and relative abundance of M^+ and prominent fragments are listed.

Table II. Recovery of Metabolites Added to Urine

metab	range, μg/mL	*n*	mean	SD	CV
DPT	40–160	19	125.7	23.3	18.6
DPDT	40–160	19	81.9	12.5	15.2
DCA	20–160	23	101.6	13.4	13.2
MCA	20–160	23	103.3	15.1	14.7
DMM	40–160	19	126.5	22.2	17.6
DPT[a]	40–267	16	87.7	29.3	33.4
DPDT[a]	40–267	16	62.8	24.2	38.5
DCA[a]	40–267	16	125.0	41.2	33.0
MCA[a]	40–267	16	76.3	25.5	33.4
DMM[a]	80–267	8	20.5	4.3	20.8

[a] The samples are hydrolyzed with Glusulase.

Table III. Recovery of Metabolites Added to Feces (Samples Treated with Glusulase)[a]

metab	*n*	mean	SD	CV
DPT	10	66.1	9.7	14.7
DPDT	10	38.2	11.3	29.6
DCA	10	34.7	5.1	14.5
MCA	10	23.1	3.5	15.0
DMM	10	18.0	4.7	26.1

[a] Range 2–20 μg/g.

Representative GLC/NPD chromatograms of urine extracts are shown in Figure 1. On the capillary GLC the compounds are well-resolved, eluting between 3.0 and 11.7 min. The retention times for DPT, DPDT, DCA, MCA, and DMM were 3.0 and 4.0, 4.8, 10.0, 10.4, and 10.8 and 11.7 min, respectively.

In Table I are listed the metabolites together with GLC retention times, molecular ion (M^+), and prominent fragments obtained from the mass spectrometric analysis. As can be seen, both compounds I and V give rise to two GLC peaks. Inspection of the mass spectra revealed an oxygen- and a sulfur-methylated derivative, respectively, of the two compounds. The characteristics of the fragmentation patterns for the different methylated metabolites are suggested in Figure 2. When the molecular fragments in Figure 2 are compared with the corresponding fragments in Table I, a difference in molecular weight of one may occur in some fragments. This is assumed to account for uptake or loss of a proton for stabilization purposes of the resulting fragment.

Table IV. Linear Regression (Least-Squares Fit) Data for Calibration Curves of Metabolites in Urine (Peak Heights Plotted on the y-Axis and Metabolite Concentrations (μg/g, μg/mL) on the x-Axis)[a]

metab	range peak ht	range, μg/mL	slope	intercept	correln coeff (r^2)
DPT	1000–11000	20–160	73.42	-231.48	0.986
DPDT	1200–18000	20–160	118.30	-1832.83	0.991
DCA	700–6700	20–160	43.57	-131.83	0.992
MCA	600–6900	20–160	45.31	-210.22	0.987
DMM	400–8100	20–160	57.82	-890.83	0.970
DPT[b]	4000–31000	20–267	115.19	-838.07	0.982
DPDT[b]	2500–21000	20–267	79.12	-1230.93	0.945
DCA[b]	5300–30000	20–267	105.24	1130.56	0.982
MCA[b]	1300–15000	20–267	58.04	-162.49	0.978
DMM[b]	300–1800	20–267	8.18	-246.33	0.912
DPT[b]	900–20000	2–40	505.68	104.80	0.999
DPDT[b]	1300–14000	2–40	314.62	806.27	0.989
DCA[b]	1700–32000	2–40	791.27	710.09	0.993
MCA[b]	400–15000	2–40	385.38	-809.20	0.995
DMM[b]		2–40			

[a] The first and second calibration curves are prepared from urine samples diluted 1:10 with water, as described in the procedure. In the third calibration curve, urine:water = 1:2. [b] The samples are hydrolyzed with Glusulase.

Table V. Linear Regression (Least-Squares Fit) Data for Calibration Curves of Metabolites in Feces (Peak Heights Plotted on the y-Axis and Metabolite Concentrations (μg/g, μg/mL) on the x-Axis) (Samples Hydrolyzed with Glusulase)[a]

metab	slope	intercept	correln coeff (r^2)
DPT	3013.10	-2280.87	0.985
DPDT	1631.89	-1753.74	0.929
DCA	1729.26	-1686.93	0.973
MCA	1113.74	-1121.13	0.979
DMM	652.60	-905.51	0.982

[a] Range 2–20 μg/g.

Recoveries of the metabolites from spiked urine and feces are shown in Tables II and III, respectively. Table IV shows the linearity of the calibration curves for urine, with the metabolite concentrations covering a 100-fold range. In Table V, the same results are shown for feces in the concentration range of 2–20 μg/g. In order to enhance the sensitivities, the urine to water volume ratio or the volume of feces extract at the beginning of the procedure may be increased. However, since the outcome of the method is dependent on the chemical conditions, i.e. ionic strength, in the sample mixture, separate calibration curves must be made following changes in the analytical procedure, as shown in Table IV.

The mean recoveries of the different metabolites from urine not treated with Glusulase varied from 82 to 127% and had variation coefficients (CV) below 20 (Table II). For quantification of DPT and DMM, the sum of the chromatographic responses of the S- and O-methylated metabolites was used. Since different detector responses are probable of the S- and O-methylated derivatives of DPT and DMM, differences in the ratio of the two derivatives in the standards and the processed samples, respectively, may contribute to the deviations in the recoveries seen for these metabolites.

When the metabolites were hydrolyzed with Glusulase, the recoveries of all metabolites except DCA were reduced, while the CVs were increased. The reduction in the recoveries is probably due to hydrolysis of the compounds during the incubation with Glusulase. The increased recovery of DCA may possibly partly be explained by hydrolyzing of MCA to DCA during the incubation. The mean recoveries of the metabolites added to feces hydrolyzed with Glusulase ranged from 18 to 66%; however, even at the lowest recovery the CV was within 30. No improvement of the recoveries was obtained by use of other solvents or volumes of solvents in preparing of the feces

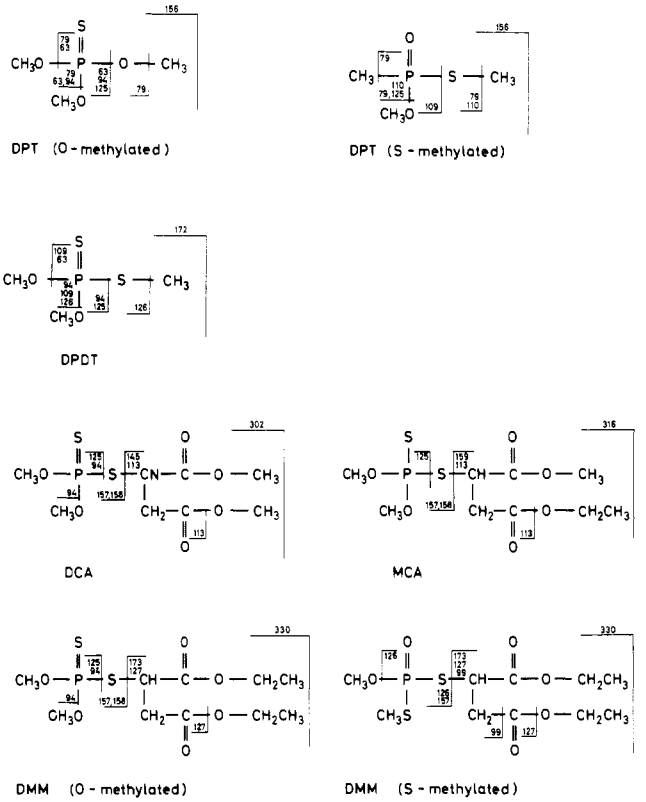

Figure 2. Suggested fragmentation pattern of the methylated main metabolites of malathion.

extracts. However, the method was considered acceptable for the purpose of metabolism studies of malathion, since feces is not an important excretion route for the metabolites in mammals.

The differences in the recoveries of the metabolites from both urine and feces may be due to differences in the chemical and physical properties of the compounds. The CVs for the repeatability of the recovery of the metabolites from urine and feces were below 14 and 20, respectively, when 12 parallel spiked samples were carried through the procedure. Most of the metabolites exhibited a satisfactory linear response, with a correlation coefficient approaching unity.

The minimum proficiency levels for the quantification of DPT, DPDT, DCA, MCA, and DMM were 2 μg/mL in urine and 2 μg/g in feces, respectively. Lowest detectable levels for screening in urine of DPT, DPDT, and DMM were 1 and 0.2 μg/mL of DCA and MCA, respectively. In

feces, the lowest detectable sensitivity for screening of DPT, DPDT, DCA, and MCA was 0.5 and of DMM was 1.5 μg/g, respectively.

This is a simple and rapid method for the isolation and quantification of the major metabolites of malathion from biological samples. The metabolites are isolated by liquid–solid extraction using anionic trimethylaminopropyl-bonded silica cartridges and quantified by GLC/NPD. The complementary use of capillary GLC–MS unambiguously confirms presumptive peaks identified by capillary GLC–NPD. Compared to older methodologies, the present procedure has the advantages of minimal handling time, clean sample extracts, and no need for concentration of the extract. The method requires only small volumes of sample and organic solvents, and the glassware requirements are also minimal. The analysis time is short, and there is minimal interference from endogenous compounds in both urine and feces. The method has been successfully used for the assay of biological samples following administration of malathion to sheep, as well as for the determination of the metabolites of malathion in different in vitro studies. With minor adjustments, the method should be applicable to most biological sample matrices.

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Registry No. I, 1112-38-5; II, 756-80-9; III, 1190-28-9; IV, 1190-29-0; V, 1116-04-7.

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Elimination of Intravenously Injected Malathion in Sheep

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The metabolism and excretion of the organophosphorus insecticide malathion were studied after a single intravenous injection of 10 mg/kg body weight of malathion to sheep. A rapid decrease in the plasma concentration and an extensive degradation of malathion to inactive metabolites were observed. Of the administered dose, 81% was eliminated within 24 h, mainly in urine. The major metabolites of malathion were malathion α-monoacid and malathion diacid in both urine and feces.

The organophosphorus compound malathion, *O,O*-dimethyl *S*-(1,2-dicarbethoxyethyl) phosphorodithioate, has a relatively low mammalian toxicity and is widely used both as an agricultural insecticide and as an agent against ectoparasites in various species of livestock and poultry, as well as in humans. Malathion is activated to malaoxon, which is responsible for the toxic effects through inhibition of cholinesterases (Hayes, 1982). The low toxicity of malathion to mammals is due to extensive degradation to inactive metabolites. The most abundant metabolites of malathion, malathion α-monoacid (MCA) and malathion diacid (DCA), are products of the hydrolytic activity of carboxylesterases (Chen et al., 1969; Bradway and Shafik, 1977). Additional metabolites arise from hydrolysis at the P–S and S–C linkages, giving *O,O*-dimethyl phosphorothioate (DPT) and *O,O*-dimethyl phosphorodithioate (DPDT), respectively, and by demethylation, giving desmethylmalathion (DMM) (Bhagwat and Ramachandran, 1975; Nomeir and Dauterman, 1978).

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