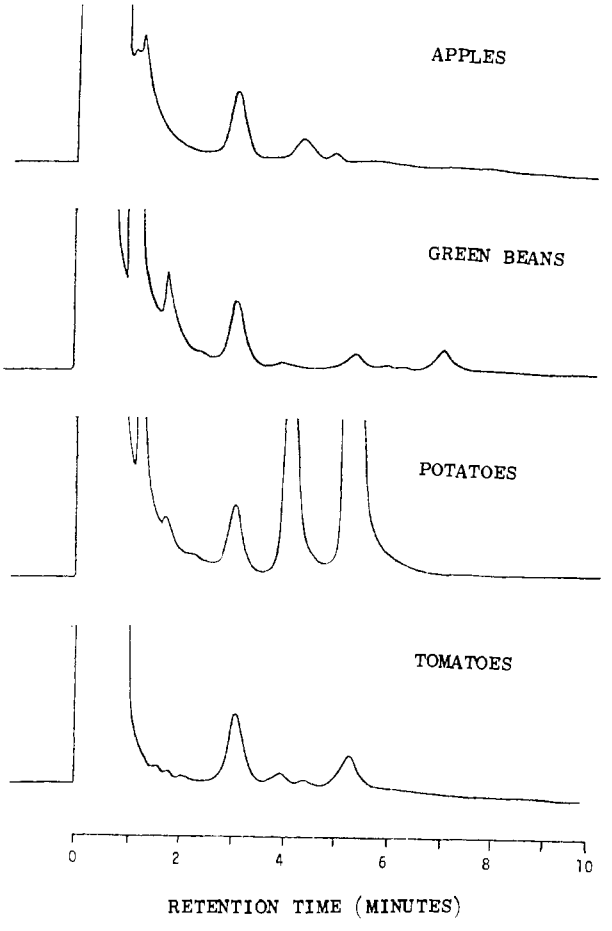

Figure 3. Gas-liquid chromatograms of derivitized apple, green bean, potato, and tomato extracts fortified with ETU at 0.05 ppm.

derivative. It was further evident from an abundant parent ion peak at m/e 260 that the *S*-(*m*-trifluoromethylbenzyl) derivative survived GLC without decomposition.

Because the electron-capture detector is nonspecific, confirmation of positive ETU determinations using an element sensitive detector was also investigated. Consequently, it was observed that the N-trifluoroacetylated *S*-(*m*-trifluoromethylbenzyl)-ETU flame photometric detector (S-mode) response was comparable to that of the *S*-(*m*-trifluoromethylbenzyl)-ETU electron-capture detector response and positive electron-capture detection of the latter could be confirmed by flame photometric (S-mode) analysis of the former. GLC–mass spectrometry confirmed the N-trifluoroacetylated *S*-(*m*-trifluoromethylbenzyl)-ETU peak and indicated a breakdown pattern analogous to Newsome's N-trifluoroacetylated *S*-benzyl ETU derivative.

ACKNOWLEDGMENT

The author wishes to thank Floyd Wood, Agriculture Canada, Fredericton, New Brunswick, Canada, for helpful discussions and also S. M. Skinner and Roy Greenhalgh of the Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario, Canada, for the NMR and GLC–mass spectral determinations.

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Received for review May 27, 1976. Accepted August 30, 1976.

Extraction and Recovery of Organophosphorus Metabolites from Urine Using an Anion Exchange Resin

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Urinary metabolites of organophosphorus pesticides are extracted quantitatively from urine by using an ion exchange resin. The metabolites were subsequently derivatized on the resin for FP–GC analysis. Several ion exchange resins and a variety of methods for the recovery of the metabolites from the resin were investigated. When used in coordination with a previously developed method, higher recoveries and decreased gas chromatographic interferences were obtained. Recovery data, limits of detectability, and analysis of urine samples from individuals exposed to organophosphorus pesticides are reported.

With the increasing demand for degradable pesticides, there has been an increase in the use and variety of organophosphorus pesticides. To the chemist, trying to monitor the metabolites of these pesticides, this situation causes a twofold need for: a faster method because of the increased work load and a new method to monitor the new metabolites for which the older methods were not intended. The work to develop a method of analysis for the

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Table I. A List of Standard Metabolites and Their Derivatives

Standard	Abbrevn	Derivative(s)
O,O-Dimethyl phosphate Na^+	DMP	*O,O*-Dimethyl *O*-amyl phosphate
O,O-Diethylphosphoric acid	DEP	*O,O*-Diethyl *O*-amyl phosphate
O,O-Dimethyl phosphorothioate K^+	DMTP	*O,O*-Dimethyl *O*-amyl phosphorothionate
	DMPTh	*O,O*-Dimethyl *S*-amyl phosphorothiolate
O,O-Diethyl phosphorothioate K^+	DETP	*O,O*-Diethyl *O*-amyl phosphorothionate
	DEPTh	*O,O*-Diethyl *S*-amyl phosphorothiolate
O-Methylphenylphosphonic acid	MPPn	*O*-Methyl *O*-amyl phenylphosphonate
O-Methyl phenylphosphonothioate	MPTPn	*O*-Methyl *O*-amyl phenylphosphonothionate
	MPPnTh	*O*-Methyl *S*-amyl phenylphosphonothiolate
Phenylphosphonic acid	PPn	*O,O*-Diamyl phenylphosphonate

alkyl phosphate metabolites of organophosphorus pesticides began some time ago. St. John and Lisk (1968) developed a method for the analysis of hydrolytic metabolites in cow urine. This method was modified and improved by Shafik et al. (1973), by extracting the metabolites from acidified urine with acetonitrile/ether. Then the metabolites were derivatized with diazopentane for gas chromatography. However, the increase in the variety of organophosphorus pesticides has brought about a new class of metabolites—the phosphonates. When trying to extract the phosphonate metabolites using acetonitrile/ether, it was found that only a small fraction of the metabolite could be recovered. Plapp and Casida (1958) had used ion-exchange resins to separate mixtures of hydrolysis products of organophosphorus pesticides. This procedure suggested the use of ion-exchange resins for the extraction of organophosphorus metabolites from urine.

The experimental work reported in this paper investigates the recovery of seven organophosphorus metabolites using an ion-exchange resin to extract the metabolites from urine. Four of the metabolites are alkyl phosphates derived from most of the common organophosphorus pesticides. The other three are phosphonate metabolites of leptophos.

EXPERIMENTAL PROCEDURE

Standards. The dialkyl phosphate standards (Table I) were supplied by American Cyanamid Corporation. The phenyl phosphonate standards were supplied by Velsicol Chemical Corporation.

The working solutions used for spiking urine samples were prepared by diluting 1 mg/ml stock solutions to 0.01 mg/ml with acetone. The stock solutions, with the exception of *O,O*-dimethyl phosphate Na^+ (DMP) were also made with acetone. Since DMP is not soluble in acetone at 1 mg/ml, the stock solution was prepared in distilled water.

Reagents. The diazopentane reagent was prepared as described by Shafik et al. (1973). Pesticide quality acetone was used throughout the investigation. The ion-exchange resin was CG-400, 100–200 mesh (Mallinckrodt Chemical Works).

Equipment. A Tracor, Model 222, gas chromatograph (GC) was used for the separation and quantification of the derivatized metabolite. The detector was a flame photometric detector equipped with a spectrum 1020 noise filter and a variable voltage power supply (Power Designs Inc.) operated in the phosphorus mode. The column used was a 6 ft × 0.5 in. o.d. × 4 mm i.d. glass column, packed with 5% OV-210 on 80/100 mesh Gas-Chrom Q. For the alkyl phosphates, the GC operating conditions of temperature and flow were: column oven, 140 °C; inlet, 200 °C; and detector, 175 °C; nitrogen carrier, 40 ml/min; hydrogen, 50–60 ml/min; and air, 80–90 ml/min. For the phosphonates, the column oven temperature was elevated to 160 °C. The samples were concentrated with a nitrogen evaporator equipped with a water bath maintained at 40 °C (Organomation Association). Disposable Mohr pipets, size 5 ml, were used for the ion-exchange columns.

Method. The ion-exchange column was prepared by weighing out 1 g of CG-400 resin and making a slurry in 0.1 N HCl. The slurry was added to a 5-ml disposable pipet with a plug of glass wool in the tip. The column was rinsed with 5 ml of 0.1 N HCl and approximately 50 ml of distilled water.

The urine was pretreated with acetone to precipitate some of the interfering compounds. One milliliter of urine was pipetted into a 13-ml, glass-stoppered, centrifuge tube and 10 ml of acetone was added. The solution was mixed well and centrifuged. The supernatant was transferred to the ion-exchange column, while being careful to avoid any particles of the residue. The residue was rinsed with 2 ml of acetone and centrifuged, and the supernatant was transferred to the column. The column was allowed to drain as much as possible. Then by using a rubber suction bulb, the ion-exchange resin was blown out of the column into a 15 × 150 mm culture tube. The empty column was rinsed with approximately 1 ml of acetone which was added to the culture tube. To aid in the removal of the metabolites from the ion-exchange resin, 0.05 ml of 6 N HCl was pipetted into the culture tube, which was allowed to stand for 1 h, with occasional stirring of the resin.

After the 1-h waiting period, the diazopentane reagent was slowly added to the resin in the culture tube until there was a persistent yellow color in the supernatant. (A large excess of diazopentane was avoided since it is a major source of background interference.) To allow the reaction to proceed after the addition of the diazopentane, the tube was set aside for at least 1 h, but with occasional stirring of the solution. If, at any time during this period, the yellow color disappeared, more diazopentane was added.

To quantify the derivatized metabolites, the supernatant was transferred to a 13-ml graduated centrifuge tube, and the resin was washed with small portions of acetone until a total volume of 10 ml was obtained. This solution was then injected into the GC and compared to standards. If the level of metabolites appeared to be too low for quantification, the sample was then concentrated and taken through silica gel fractionation. This procedure, described in the method of Shafik et al. (1973), is used to remove interferences and quantify levels as low as 0.01 ppm.

To calculate recoveries, urine samples were spiked with metabolites before the acetone pretreatment and carried through the procedure. Standards containing identical amounts of metabolites were pipetted into 1 ml of acetone, and 1 drop of 2 N HCl was added to convert the salts to the corresponding free acids. The standards were allowed to stand for 1 h before being derivatized with diazopentane. The standards were then diluted to 10 ml with acetone and injected into the GC. After any interfering peaks found in the control urine were subtracted from the spiked urine,

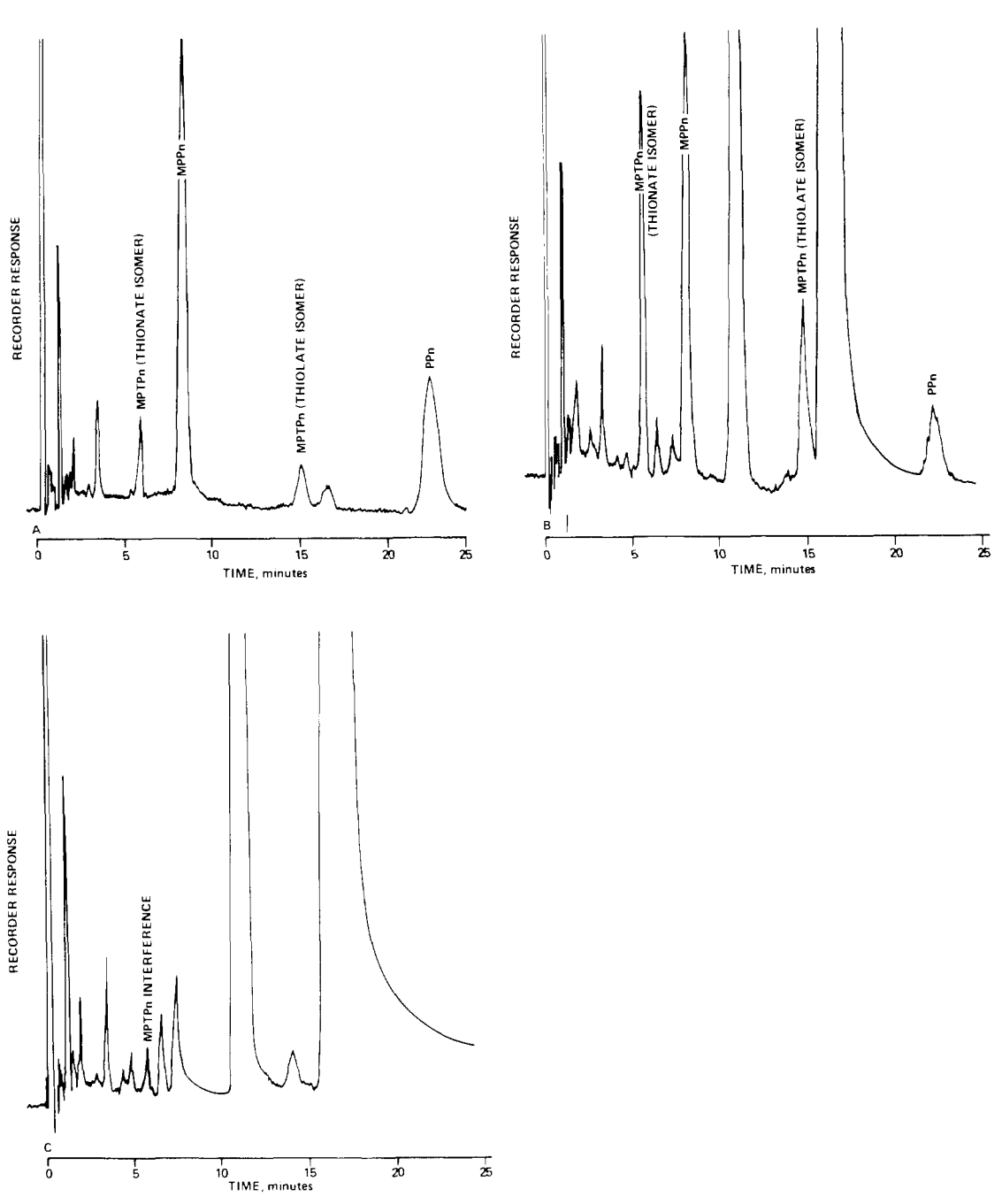

Figure 1. Chromatograms of phosphonate samples injected into a GC without cleanup: (A) phenylphosphonate standards, 0.5 ng each; (B) extract of urine from rats fed leptophos at 0.01 LD_{50}; (C) control rat urine.

the peak heights of the standard and the spiked urine samples were compared.

RESULTS AND DISCUSSION

One of the major factors influencing the recovery of the metabolites from urine was found to be the urine:acetone ratio used to precipitate the interfering compounds. In order to determine the optimum ratio, several urine: acetone ratios were investigated for each metabolite. One milliliter of urine was used throughout the experiment and the volume of acetone was varied from 3 to 10 ml. As shown in Table II, no single ratio was optimum for all alkyl phosphates and phosphonates. The lower amounts of acetone seem to favor some compounds, while the higher amounts seem to be best for others. When a specific alkyl phosphate is being sought, the urine:acetone ratio which gives the best recovery for that compound should be used. However, for a general screening method, a compromise ratio must be selected. The 1:10 ratio, selected for general use in this laboratory, also gave cleaner chromatograms

Table II. Comparison of Percentage Recovery with Various Urine:Acetone Ratios

Compd	Av recovery (N)[a] for ratios		
	1:3	1:5	1:10
DMTP	*b*	90 (2)	39 (20)
DETP	*b*	81 (2)	77 (20)
DMP	*b*	85 (3)	85 (20)
DEP	*b*	65 (3)	105 (20)
DMPTh	*b*	Not analyzed	95 (20)
DEPTh	*b*	Not analyzed	87 (20)
MPTPn	Interference	Interference	74 (2)
MPPn	92 (6)	92 (6)	44 (2)
PPn	71 (6)	82 (6)	79 (2)

[a] N represents the number of determinations. [b] Background too high to be useful.

Table III. Recovery, Variability, and Detection Limits of Standard Derivatives

Compd	% recovered for spiking level 0.1 ppm	0.5 ppm	1.0 ppm	Limit of detection without cleanup, ppm
DMTP	51	36 ± 14	42 ± 13	0.1
DETP	87	77 ± 10	76 ± 11	0.1
DMP	74	85 ± 20	85 ± 15	0.05
DEP	97	106 ± 15	105 ± 13	0.05
DMPTh		97 ± 23	93 ± 8	0.1
DEPTh		87 ± 9	87 ± 7	0.1
MPTPn			74	0.15
MPPn	97 ± 7	95 ± 14	90 ± 8	0.04
PPn	87 ± 11	86 ± 18	79 ± 22	0.07

than the other ratios that were tried.

The pH of the urine/acetone mixture was investigated and found to have little effect on the recovery of the metabolites. The time required for complete derivatization was also checked and it was found that, while most of the reaction was complete within 1 h, increased yields could be obtained with overnight waiting periods.

The recovery data shown in Table III were obtained from spiked urine samples without silica gel cleanup. The recovery data are based on a 1:10 urine:acetone ratio for the alkyl phosphates, and on a 1:5 ratio for the phosphonates. The low recovery of *O,O*-dimethyl *O*-amyl phosphorothionate (DMTP) is due to an interfering peak that seems to come from the diazopentane reagent. However, since the other isomer, *O,O*-dimethyl *S*-amyl phosphorothiolate (DMPTh) can be quantified, the original level of *O,O*-dimethyl phosphorothioate can be determined (Shafik et al., 1970).

Of those investigated, the only compound that could not be quantitatively determined was *O*-methylphenyl phosphonothioate (MPTPn). There was an interfering peak that could not be resolved on the GC or removed by silica gel cleanup. The interference, which comes from the urine, can be reduced enough to allow qualitative determinations by using a 1:10 urine:acetone ratio.

The recovery of the metabolites seemed to vary from one urine sample to the next. So, to check the variability of the method, morning and afternoon urine samples were obtained from five people. These samples were spiked with alkyl phosphates at 0.5 and 1.0 ppm. As shown in Table III, the variation, which was possibly due to the variation in the salt content of different urines, was a little more than desirable.

The variability of urine is also a major factor influencing the limit of detectability, which is below 0.1 ppm for the alkyl phosphates. In some urine samples, where the level of interference is low, the sample may be evaporated to 1 ml for detection of very low levels of metabolites. If even lower limits of detection are required, or if the interference level is too high, the sample must be carried through silica gel fractionation. The limit of detectability for the phosphonates ranged from 0.04 ppm for the *O*-methylphenylphosphonic acid (MPPn) to 0.15 ppm for phenylphosphonic acid (PPn). Cleanup was found to be of little help with the phosphonates because the interference is not removed by silica gel.

Figure 1 shows chromatograms of the phosphonate standards, urine from leptophos-treated rats, and control urine. The peak which interferes with the determination of MPTPn can be seen in the control urine. Figure 2 shows chromatograms of the alkyl phosphate standards, spiked urine, control urine, and urine from a dicrotophos exposure

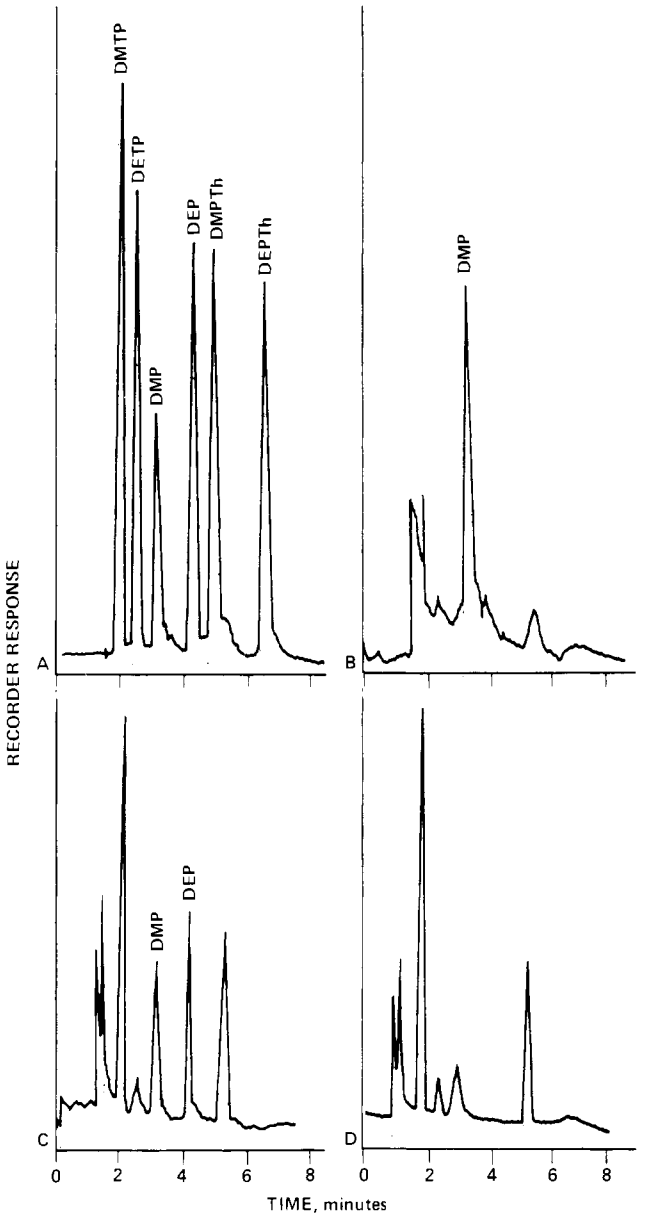

Figure 2. Chromatograms of alkyl phosphates injected into a GC without cleanup: (A) alkyl phosphate standards, 0.5 ng each; (B) urine extract from dicrotophos poison case; (C) extract of human urine spiked with 0.5 ppm of DMP and DEP; (D) control human urine.

case. The control urine shown here has a peak that interferes with the determination of DMP. However, the level of interference was generally low enough to allow quantification of levels above 0.1 ppm. With a highly efficient 5% OV-210 column (5000 theoretical plates), it was possible to obtain baseline separation of the alkyl phosphates. With less efficient GC columns, the silica gel fractionation was required to separate the phosphate derivatives.

This method has been applied to media other than urine, and alkyl phosphates have been determined in whole blood, serum, stomach contents, and even in liver from poison cases. This makes the method particularly valuable in clinical labs where the rapid classification of poisoning agents is essential. In conclusion, this method provides a rapid, sensitive screening procedure for the analysis of organophosphonate metabolites and a significant short cut in the analysis of alkyl phosphate metabolites. Using this method, a minimum of 8 to 10 samples per day can be

analyzed, making the method better adapted to the number of samples encountered in a monitoring program.

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Received for review July 19, 1976. Accepted September 24, 1976.

Diphenadione Residues in Milk of Cattle

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The mammillary transfer of diphenadione (2-(diphenylacetyl)-1,3-indandione) into the milk of treated cows is an important health aspect of the use of this systemic anticoagulant in vampire bat (*Desmodus rotundus*) control. Milk of cows dosed intraruminally with diphenadione at the recommended 1-mg/kg level did not contain detectable levels of residues, whereas cows treated with 2.75 mg/kg contained 21.3 ppb or less at 12, 24, and 48 h posttreatment. There were no residues in samples after 72 h. In nursling calves the prothrombin clotting time did not change and there were no detectable residues in their blood plasma. It appears that the mammillary transfer of diphenadione, like that of other anticoagulants, is dose dependent. Our data indicate that it is safe to consume milk from cows dosed at the recommended rate of 1 mg/kg. Even if cows are accidentally given 2.75 times that amount, and milk collected within 72 h after treatment is consumed, there are no apparent health hazards involved.

Diphenadione (2-(diphenylacetyl)-1,3-indandione) is a systemic anticoagulant that has been found to be effective in killing vampire bats (*Desmodus rotundus*) that consume blood from cattle treated intraruminally. One feeding is effective if it takes place within 3 days after cattle are given a 1 mg/kg dose (Thompson et al., 1972). This promising control method could save the Latin American cattle industry up to $250 million annually (Greenhall, 1970).

Currently, the major emphasis is to determine how the diphenadione treatment affects livestock and if there is a potential health hazard to humans who consume meat and milk from treated animals. Thompson et al. (1972) reported that a moderate increase in clotting time of plasma prothrombin is the only observable sign of intoxication in adult cattle. Later, residues were found in liver (about 0.15 ppm) and kidneys (about 0.08 ppm) of treated animals at 30 to 90 days posttreatment but not in heart, brain, muscle from the hindquarter, fat, or blood plasma (Bullard et al., 1976). Calculations and secondary hazard tests in albino rats indicated that these levels are of no danger to humans that consume liver and kidneys from treated animals.

Other anticoagulants have been found to undergo mammillary transfer. Their appearance in the milk of treated subjects appears to be dose dependent. The administration of massive doses of dicumarol (Field, 1945) or warfarin (Blumberg et al., 1960) to lactating rats produced hypoprothrobinemia in suckling young. However, dicumarol administered prophylactically to 125 nursing mothers did not affect prothrombin activity of the infant (Brambel and Hunter, 1950). Furthermore, in 4000 mothers given oral anticoagulants prophylactically, all of the breast-fed infants remained asymptomatic throughout the period of therapy (Fries et al., 1957). Warfarin could not be found by chemical analysis in the milk of two women that were on a therapeutic anticoagulant regimen (O'Reilly and Aggeler, 1970).

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However, since there are no reports concerning diphenadione in milk, it was imperative that we determine if mammillary transfer occurs in treated cows. Cows were tested at 2.75 mg/kg as well as 1 mg/kg so that dose-dependent responses could be observed in case of accidental overdosing.

EXPERIMENTAL SECTION

Treatment of Animals and Collection of Samples. Three lactating cows were given the recommended 1-mg/kg intraruminal doses of diphenadione and three others were given 2.75 mg/kg. Each cow had a nursling calf. A Carbopol 941 aqueous suspension of the compound was injected with a pistol grip automatic syringe (Vaco HL 013700) having a 14 gauge, 1.5-in. disposable needle. A control cow (also with nursling calf) received a "sham" injection of physiological saline.

Samples of milk and blood from each cow and blood from each calf were collected immediately pretreatment and at 12, 24, 48, 72, 96, 120, and 144 h posttreatment. Ten-milliliter blood samples were obtained by venipuncture from the jugular vein. Prothrombin clotting times (Quick, 1935) were determined immediately after collection. All samples were stored at −12 °C until analyses could be conducted.

Residue Analysis. A gas–liquid chromatographic (GLC) procedure reported earlier in this journal was used for analysis of all milk and blood samples (Bullard et al., 1975). The only difference in analysis of milk and blood is in the sample preparation. The plasma fraction of venous blood is extracted with acetone, and proteins are removed by centrifugation. In milk, most of the water is removed through evaporation and then the residue is mixed with anhydrous sodium sulfate and extracted with acetone. The acetone extract in both cases is processed the same way through the remainder of the procedure. Diphenadione cannot be analyzed by GLC directly but is oxidized to benzophenone which chromatographs readily and is sensitive to electron-capture detection.

An Aerograph 1520B gas chromatograph equipped with a 0.0625-in. i.d. injection port liner and a tritium foil