Toxicological Effects of Pesticides on Rumen Function *In Vitro*

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Twelve pesticides were studied *in vitro* to ascertain their effect upon rumen microbial activity based upon the following criteria: dry matter disappearance, volatile fatty acid production, and alterations in rumen ciliated protozoal numbers. Results indicated that relatively high concentrations (500 mcg per ml) of pesticides were tolerated by rumen microorganisms without deleterious effects to rumen function. Significant decreases ($P < 0.05$) in dry matter disappearance were found with all pesticides tested with the exception of malathion and 2,3,6-TBA. 2,4,5-T was found to be most toxic of the pesticides evaluated and caused significant ($P < 0.05$) decreases in dry matter disappearance and volatile fatty acid production.

It is now well recognized that the inherent problems associated with pesticide use include accumulation and secretion of either the parent compound or its degradation products in edible tissues (Biddulph *et al.*, 1950; Claborn *et al.*, 1962; Fries and Kane, 1967; Laben, 1968; Rumsey *et al.*, 1966; Waldron *et al.*, 1968), milk (Bateman *et al.*, 1953; Crosby *et al.*, 1967; Laben *et al.*, 1966; Zweig *et al.*, 1963), and other human foods. Research in the pesticide area within the past several years has primarily dealt with delineating the routes, as well as rates, of pesticide excretion products in urine and feces of ruminants (Bakke *et al.*, 1969; Feil *et al.*, 1969; Gutenmann *et al.*, 1968; O'Brien *et al.*, 1961; Robbins *et al.*, 1968, 1969; St. John and Lisk, 1969), whereas some of the earlier work was concerned with pesticide degradation in the rumen.

Cook (1957) was perhaps the first to suggest that rumen liquor played an active role in hydrolyzing organophosphates, particularly parathion. Additional evidence by Cook (1957) indicated that metabolism of parathion by rumen microorganisms accounted for its apparent lack of toxicity to cattle. Similar implications have been attributed to DDT since Fries (1968) demonstrated a high conversion efficiency of ^{14}C-DDT to less toxic DDD by rumen microorganisms. Ahmed *et al.* (1958) and Plapp and Casida (1958) demonstrated that other organophosphates, malathion and trolene specifically, undergo hydrolysis to varying degrees in the presence of rumen liquor.

Certain chlorinated hydrocarbon-, organophosphate-, and carbamate-containing insecticides were shown by Williams *et al.* (1963) to stimulate gas production *in vitro* by rumen holotrich protozoa, whereas these compounds had no appreciable effect when rumen bacteria served as the inoculum source. More recent work by Williams *et al.* (1968) indicated that propazine, atrazine, or simazine did not inhibit or stimulate gas production, nor was $^{14}CO_2$ production detected from protozoal metabolism.

The importance to the host of the microbial population harbored in the rumen is well known. However, little attention has been given to the events occurring in the rumen when pesticide-contaminated forages are ingested by ruminants.

Therefore, the studies reported herein were initiated to investigate the effect of a variety of pesticides upon *in vitro* rumen fermentation. Parameters evaluated were *in vitro* dry matter disappearance (IVDMD), volatile fatty acid (VFA) production, and changes in protozoal (ciliate) numbers.

EXPERIMENTAL PROCEDURE

Pesticide Compounds. Insecticides and herbicides used in this study with their source of supply are listed below. Grade and purity of compounds are indicated in parentheses.

BROMACIL. 5-bromo-3-*sec*-butyl-6-methyluracil, Allied Chemical Co. (technical, 95%).

DICAMBA. 3,6-dichloro-*o*-anisic acid, Velsicol Chemical Co. (analytical, 99.8%).

DIURON. 3-(3,4-dichlorophenyl)-1,1-dimethylurea, Allied Chemical Co. (technical, 95%).

DDT. 1,1,1-trichloro-2,2-bis-(*P*-chlorophenyl)-ethyl phosphorodithioate, American Cyanamid Co. (analytical, 99.3%).

SEVIN. 1-naphthyl-N-methylcarbamate, Union Carbide Co. (analytical, 99.9%).

SIMAZINE. 2-chloro-4,6-bis(ethylamino)-s-triazine, Geigy Chemical Co. (technical, 99.3%).

TORDON. 4-amino-3,5,6-trichloropicolinic acid, Dow Chemical Co. (analytical, 99%).

TOXAPHENE. Octachlorocamphene, Hercules Chemical Co. (68% chlorine content).

2,3,6-TBA. 2,3,6-trichlorobenzoic acid, E. I. Du Pont de Nemours & Co. (60% as 2,3,6-TBA).

2,4-D. 2,4-dichlorophenoxyacetic acid, Miller Products. (technical, 98%).

2,4,5-T. 2,4,5-trichlorophenoxyacetic acid, Miller Products. (technical, 98%).

Stock solutions (10,000 mcg/ml) of the various pesticides were dissolved in appropriate organic solvents (benzene, chloroform, or ethanol) and known volumes dispensed into fermentation vessels containing 1 g of forage (fescue). Prior to inoculation, the solvent phase was evaporated. Concurrent studies with solvents used in this study had no effect on IVDMD, VFA, or protozoal numbers.

Inoculum Source. Rumen liquor for *in vitro* fermentation

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Table I. Influence of Pesticides on Percent *In Vitro* Dry Matter Disappearance

Treatment	Pesticide levels, mcg/ml					
	0[e]	100[e]	250	500	750	1000
	%					
Insecticide						
DDT	30.80	30.92[a]	29.69[ab]	28.05[ab]	26.02[b]	25.74[b]
Malathion	28.92	29.17	29.32	28.67	27.60	26.51
Sevin	28.97	29.91[a]	28.40[ab]	25.92[abc]	25.07[bc]	22.38[c]
Toxaphene	27.95	28.51[a]	27.85[ab]	25.71[abc]	22.37[cd]	19.17[d]
Herbicide						
2,3,6-TBA	28.97	30.51	29.28	28.78	27.97	26.02
Tordon	30.02	28.83[a]	27.58[ab]	25.01[abc]	23.74[bc]	22.02[c]
Dicamba	29.40	30.11[a]	27.31[ab]	25.89[ab]	24.78[ab]	23.26[b]
Diuron	29.03	27.79[a]	24.55[a]	18.24[b]	16.61[b]	15.09[b]
2,4-D	29.49	28.38[a]	25.48[ab]	23.44[bc]	21.80[bc]	19.58[c]
Simazine	29.49	28.98[a]	25.76[ab]	23.30[bc]	20.83[cd]	18.62[d]
Bromacil	28.50	28.41[a]	27.74[ab]	25.02[abc]	22.84[cd]	18.50[d]
2,4,5-T	29.07	27.80[a]	24.60[a]	17.76	10.77[b]	8.28[b]

[a,b,c,d] Means sharing common superscripts or with no superscripts among the five levels and within each treatment are not significantly different ($P > 0.05$) according to Duncan's New Multiple Range Test.
[e] Not statistically different at the 0.05 level.

studies was obtained from a fistulated sheep maintained on a forage diet *ad libitum*. Rumen liquor was withdrawn via a dosing syringe from the ventral-caudal sac, immediately taken to the laboratory, and strained through four layers of cheesecloth to remove the large particles of solid ingesta. The strained rumen liquor was then placed into a 2-l. separatory funnel and incubated for 1 to 1.5 hr at 39° C so that the finer forage particles rose to the top. The resulting semiclarified rumen liquor (lower layer) was withdrawn and served as the inoculum source.

Fermentation Conditions. *In vitro* fermentation studies were conducted in 100 ml Berzelius lipless beakers fitted with a No. 10 one-hole rubber stopper containing a gas release valve. To each vessel containing 1 g of forage substrate and varied amounts of pesticide (0, 100, 250, 500, 750, 1000 mcg/ml), 10 ml of inoculum, 35 ml of buffer (McDougall, 1948) and 32 mg each of urea and glucose were added. Following the addition of inoculum, the fermentation vessels were swept gently with CO_2 and stoppered with gas release valves. Incubation was allowed to proceed for 24 hr at 39° C at which time microbial activity was stopped by the addition of 1 ml of 2N H_2SO_4; the beakers were then refrigerated. Negative control vessels were acidified immediately after inoculation.

Dry Matter Determination. Contents in the fermentation beakers were filtered through 50 ml sintered glass Gooch-type crucibles (40 mm diameter disc, porosity C) and dried at 70° C for 12 to 48 hr, cooled, and weighed. Percent *in vitro* dry matter disappearance was calculated from the loss in weight as compared to unfermented controls.

Sample Preparation for Volatile Fatty Acid Analysis. Procedures employed for preparing rumen liquor for volatile fatty acid analysis were essentially those outlined by Erwin *et al.* (1961). Duplicate samples from each level (0, 100, 250, 500, 750, 1000 mcg/ml) were pooled, 10 ml aliquots combined with 2 ml of 25% *meta*-phosphoric acid, and allowed to stand at room temperature for one-half hour. The samples were then centrifuged at 12,000 rpm for 20 min and the supernatant decanted and analyzed for VFA.

Volatile Fatty Acid Analysis. An Aerograph Hy-Fi (Model 1200) gas chromatograph (Wilkins Instrument Co.), equipped with a high-temperature hydrogen flame ionization detector and a Bristol (Bristol Co.) Dynamaster recorder (Model 560) were employed. Ultrapure hydrogen was supplied by an Aerograph Elygen electrolytic hydrogen generator (Model 9652). A commercial source of nitrogen served as the carrier gas, while an air pump delivered air for the flame-ionization assembly. A commercial stainless steel column 1.524 m by 0.318 cm was used containing 20% neopentylglycol succinate (NPGS) with 2% H_3PO_4 on 60 to 80 mesh Chromosorb W as the support. Operating conditions were as follows: oven—145° C, injector—185° C, and detector—200° C, chart speed—2.54 cm per min. Flow rates of nitrogen and hydrogen were 25 ml per min and 30 ml per min, respectively. VFA were quantitated according to the procedures outlined by Baumgardt (1964).

Ciliated Protozoal Enumeration. One-milliliter aliquots of the incubation medium collected prior to addition of H_2SO_4 were placed into a Sedgwick-Rafter counting chamber, and five randomly selected fields counted under a 10× objective and 10× eyepiece. Counting was facilitated by inserting a net micrometer disc (5 mm^2, subdivided into 25 squares) into the ocular.

Statistical Analysis. The statistical design employed in this study was a 5 (level) × 12 (treatment) factorial with nine observations per treatment (IVDMD). Means for VFA and protozoal numbers represent values from 2 days in which duplicate samples were pooled on each day. Differences between the five levels of pesticide were analyzed according to the procedures outlined by Steel and Torrie (1960). Controls and level 1 (100 mcg/ml) for the 12 treatments were compared by a paired t-test.

RESULTS AND DISCUSSION

Results of insecticides and herbicides on IVDMD are presented in Table I. DDT, sevin, and toxaphene were ineffectual in causing a depression of IVDMD at the 100, 250, and 500 mcg per ml levels, but statistically significant differences ($P < 0.05$) were found in degree of IVDMD inhibition between lower (100 mcg/ml) and upper (750 and 1000 mcg/ml) levels. Toxaphene appeared to be the most inhibitory of the insecticides tested and decreased IVDMD by 34%. No significant differences ($P > 0.05$) between the five levels of malathion were found; presumably, hydrolysis of the organophosphate to a less toxic compound by the ruminal flora, as suggested by Cook (1957), may explain the small differences noted.

Of the herbicides tested, 2,4,5-T was most effective in

Table II. Effect of Herbicides and Insecticides on Volatile Fatty Acid Production and Molar Percent *In Vitro*

Treatment[e]	Pesticide levels, mcg/ml					Molar %		
	100	250	500	750	1000	C_2	C_3	C_4
	μM/ml					%		
Control[e]	...	...	...	...	...	57	33	10
DDT	87	88	89	94	91	52	37	11
Sevin	95	97	92	93	94	50	41	9
Toxaphene	93	91	86	80	75	51	40	9
Malathion	102	104	102	97	95	57	33	10
2,3,6-TBA	87	83	87	86	87	59	32	9
Tordon	96	89	97	91	92	54	37	9
Dicamba	94	94	89	84	92	62	30	8
2,4-T	83	86	90	89	85	54	36	10
Simazine	89	89	88	88	87	55	36	9
Bromacil	92	99	85	90	83	62	30	8
Diuron	93	92	83	81	86	63	29	8
2,4,5-T	91[ab]	97[a]	78[abc]	63[cd]	58[cd]	64	28	8

[abcd] Means with no superscripts or those sharing common superscripts within treatments are not significantly different ($P > 0.05$) according to Duncan's New Multiple Range Test.
[e] Control contained 89 μM/ml of VFA.

Table III. Influence of Herbicides and Insecticides on *In Vitro* Ciliated Protozoal Numbers

	Pesticide levels, mcg/ml					
	0	100	250	500	750	1000
	× 10^4/ml					
Insecticides						
DDT	9.94	10.15	8.70	8.79	8.70	9.66
Malathion	5.12	4.61	4.33	4.26	2.67	2.36
Sevin	5.78	5.37	4.22	3.95	4.88	3.26
Toxaphene	5.78	4.43	3.33	2.84	2.34	2.19
Herbicides						
2,3,6-TBA	9.44	11.97	8.99	6.91	11.48	8.99
Tordon	5.78	4.88	5.40	3.92	5.09	5.51
Dicamba	9.44	11.55	9.42	7.29	7.65	8.93
2,4-D	5.78	5.16	5.27	5.58	3.84	2.36
Simazine	5.78	6.09	5.82	4.46	5.16	5.68
Bromacil	9.44	11.10	5.58	8.69	6.69	3.11
Diuron	9.44	9.03	7.53	5.64	6.69	4.88
2,4,5-T	9.44	12.98	10.66	5.89	6.48	1.67

depressing IVDMD. Unlike the insecticides and other herbicides, 2,4-D, simazine, diuron, and 2,4,5-T depressed IVDMD at significantly lower levels, beginning at 500 mcg/ml and progressively inhibiting microbial activity as the pesticide concentration increased.

Studies conducted by Dewey *et al.* (1962) with biologically active soil showed that 2,3,6-TBA is degraded with the release of inorganic chlorides. Therefore, the action of rumen microorganisms on 2,3,6-TBA could very well be via dechlorination and thus decrease the toxicological properties of the compound.

Volatile fatty acids (VFA) arising from rumen fermentation supply the metabolic fuels for ruminants; alteration or lowered quantities of VFA, therefore, have significant implications with respect to animal performance.

The effect of pesticides on *in vitro* VFA production are presented in Table II. With the exception of 2,4,5-T, total μM/ml of VFA did not differ significantly among the five levels tested with either insecticides or herbicides. However, 2,4,5-T additions resulted in a significant decrease ($P < 0.05$) of 33 μM/ml of VFA between the 100 and 1000 mcg/ml levels. Decreases in VFA production were noted with toxaphene, but were nonsignificant.

Molar percent of acetate (C_2) and propionate (C_3) varied to some degree among insecticides and herbicides, whereas butyrate (C_4) remained relatively constant. It is interesting to note that sevin and toxaphene resulted in higher molar percent of propionate production *in vitro*. While other insecticides and herbicides were intermediate in molar percent of VFA produced, diuron and 2,4,5-T resulted in greater amounts of acetate produced. While the glc procedures employed were capable of detecting iso-butyrate, iso-valerate, and valerate, these VFA accounted for less than 1.5 μM/ml of the total acids quantitated.

The effects of insecticides and herbicides in altering ciliated protozoal numbers are shown in Table III. Generally, ciliated protozoal numbers decreased linearly from 6.66 to 4.37 × 10^4/ml when insecticides were present. In some instances, however, ciliated protozoal numbers increased at the 100 mcg/ml level, then decreased. With respect to the herbicides, 2,3,6-TBA, dicamba, simazine, bromacil, and 2,4,5-T resulted in apparent stimulation of ciliated protozoal numbers at the 100 mcg/ml level. As with IVDMD inhibition and reduction of VFA, 2,4,5-T reduced ciliated protozoal numbers drastically from 9.44 to 1.67 × 10^4/ml (82%).

Viable protozoal cells counted *in vitro* included both holotrich (*Isotricha* sp., *Dasytricha* sp.) and Entodiniomorphs (*Entodinium* sp., *Epidinium* sp., *Diplodinium* sp. and *Polyplastron* sp.) species (Hungate, 1966). Toxicity of pesticides toward protozoal cells did not appear to be species specific, however.

Correlation coefficients for the various pesticides and parameters evaluated are shown in Table IV. Pesticides that were effective in causing decreases in IVDMD, total VFA,

Table IV. Correlation Coefficients of IVDMD, VFA, and Ciliated Protozoal Numbers as Influenced by Pesticides

	r[a]	r[b]	r[c]
Insecticides			
DDT	0.092	−0.930	−0.299
Malathion	0.957	0.984	0.968
Sevin	0.747	0.595	0.354
Toxaphene	0.881	0.996	0.290
Herbicides			
2,3,6-TBA	0.293	−0.206	−0.036
Tordon	−0.140	0.299	−0.809
Dicamba	0.742	0.564	0.803
2,4-D	0.786	−0.357	0.265
Simazine	0.400	0.843	0.504
Bromacil	0.739	0.718	0.146
Diuron	0.921	0.851	0.669
2,4,5-T	0.932	0.967	0.886

[a] IVDMD with protozoal numbers. [b] IVDMD with total-VFA. [c] Protozoal numbers with total-VFA.

and ciliated protozoal numbers (toxaphene, diuron, 2,4,5-T) were highly correlated, thereby demonstrating simultaneous alterations in rumen function. High correlations were associated with malathion even though significant changes among levels did not occur; however, decreases in IVDMD, VFA, and ciliated protozoal numbers were evident.

Recent studies conducted by Williams *et al.* (1968) showed that VFA molar percent and protozoal numbers were unaltered when propazine was fed below toxic levels to sheep (5 mg/kg of body weight). Studies in our laboratory under *in vitro* conditions also indicate that simazine, a triazine, had little effect on ciliated protozoal numbers (Table III) and VFA production (Table II), although IVDMD decreased by 33%. The fact that some pesticides were effective in depressing IVDMD significantly, but not VFA or protozoal numbers, suggests that the bacterial population is affected by pesticides perhaps to a greater degree than protozoa. However, this does not preclude population shifts between bacterial species, thus offering a tentative explanation for lowered IVDMD but little change in VFA.

It has been shown with several pesticides that rumen microorganisms are capable of converting the parent compound to less toxic forms (Ahmed *et al.*, 1958; Cook, 1957; Fries, 1968; Miskus *et al.*, 1965). However, in some cases, conversion products may be more toxic than the parent compound. Heptachlor, for example, is converted to a stable toxic epoxide (Egan, 1966). Further work is needed in this area in terms of pesticide degradation, especially with compounds which, in this study, exhibited the most pronounced toxicity toward rumen microorganisms.

The results of this study indicated that high concentrations of pesticides (1000 mcg/ml) significantly effected rumen microbial processes as evidenced by the parameters evaluated. Even though high levels were toxic, there were no significant differences ($P > 0.05$) in *in vitro* forage digestibility at the lower pesticide concentrations (0, 100, and 250 mcg/ml). Since pesticide contamination of feedstuffs ingested by ruminants are usually lower than these amounts (250 ppm), it was concluded that pesticides would have a negligible effect on rumen digestibility or other associative rumen function.

The *in vitro* studies reported herein offer distinct opportunities for further study of pesticide metabolism of ruminants. Not only does it provide a rapid and convenient method for screening pesticides, but *in vitro* techniques are highly correlated with animal performance (Bowden and Church, 1962) and could lead to recommendations regarding the feeding value of pesticide contaminated forages.

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