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Biosynthesis of Hepta- and Octa-chlorodioxins in Cattle and Evidence for Lack of Involvement by Rumen Microorganisms

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Abstract

Measurements of intake and excretion of all 2,3,7,8- substituted dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) were made in cows administered pentachlorophenol (PCP) treated wood. This quantitative mass balance was conducted for the last 5 days of a 58-day dosing period. The excretion of 1,2,3,4,5,6,7,8-CDD and 1,2,3,4,5,6,7,8,9-CDD exceeded intake by factors of 1.9 and 3.7, respectively. Intake and excretion of other PCDD/Fs did not differ significantly. The initial hypothesis that the apparent synthesis of HpCDD and OCDD occurred in the rumen was not confirmed with *in vitro* fermentation of the PCP-treated wood by rumen microorganisms.

Introduction

Animal products were identified as important contributors to human background exposure in the U. S. Environmental Protection Agency reassessment of dioxins and related compounds.[1] Animal exposures to combustion emissions deposited on pasture and forage crops were considered the primary source, but later studies provided evidence that exposure to PCP-treated wood may be an important pathway of animal exposure to PCDDs and PCDFs.[2,3]

We administered PCP-treated wood to dairy cows to quantitatively understand the transfer of PCDD/F contaminants to milk and tissues.[4] A mass balance measurement was conducted at the end of the dosing period. This paper presents mass balance measurements, and the results of a followup study to determine if metabolic action by rumen microorganisms was the mechanism of the apparent biosynthesis of HpCDD and OCDD.

Materials and Methods

In Vivo Mass Balance. A detailed description of the design of the cattle study is available.[4] The study followed an animal care protocol approved by the Beltsville Area Animal Use and Care Committee. The four Holstein cows in mid-lactation were confined in stalls fitted with rubber mats and bedding was not used. Cows were milked at twelve hour intervals, the amount of milk was recorded. The cows were administered a 3.0 g of ground PCP-treated wood once per day by gelatin capsule for 58 days.

Samples for the balance study were collected during the last 5 days of the dosing period (Days 54-58). Total fecal output of each of the four cows was collected, weighed, mixed, and

sampled daily. A 5-day composite sample for each cow was prepared from the 1-day samples. Feed intake was measured each day, and a single composite sample was prepared from the five daily samples. The fecal and feed samples were dried at 60 °C and ground prior to PCDD/F analysis. Water and urine were not sampled because these materials are not important contributors to intake and excretion of 5-day PCDD/Fs.[5] A 3 L milk sample was collected from each cow at a single milking on Day 56.

In Vitro Fermentation. Fermentation studies were carried out in the laboratory to determine if rumen microorganisms were the source of apparent HpCDD and OCDD synthesis in the balance study. The test substrate was prepared by adding the same PCP-treated wood used in the cow study to ground alfalfa-orchard grass hay at the 1% level. The hay without added wood served as a control. Ruminal fluid was obtained from a Holstein cow fitted with a cannula and the fluid was mixed on an equivalent basis with artificial saliva under anaerobic conditions. Initial pH of the mixture was 6.8. Replicate samples of substrate (weight = 0.500 g, n=5) for each treatment were placed in 60 ml serum bottles with 30 ml of the ruminal fluid mixture, flushed with CO_2 and incubated in a water bath for 48 h at 39 °C.

The volume of gas produced during incubation was measured by collecting gas expressed through an hypodermic needle into an inverted, water-filled graduated cylinder. The fermentation was terminated by the addition of 1 ml of 50% H_2SO_4 and 1 ml samples were removed and filtered for analysis of volatile fatty acids by GLC. Ammonia was determined on the filtered samples by a Technicon autoanalyzer. A completely randomized arrangement of treatments was analyzed by PROC GLM (SAS 6.12) using a one-way ANOVA to determine the influence of PCP-treated wood on fermentation characteristics. The treated substrate, a 48-h control, and two each 0-h and 48-h treated samples were analyzed for PCDD/Fs.

PCDD/F Analyses. The extraction, cleanup, and quantitation of the PCDD/F homologs in the samples by high resolution gas chromotography-mass spectrometry were performed by Alta Analytical Laboratory (El Dorado Hills, CA) following USEPA methods 1613A and 8290.

Results and Discussion

Mass Balance. The results of the mass balance study are presented in Table 1. The intake values include the PCDD/Fs present in both the feed and the PCP-treated wood. Feed was an important contributor to intake only in the case of 2,3,7,8-CDD and the tetra- and penta-CDFs. Feces was the primary route of excretion of all congeners, and this route increased in importance with increased chlorination. Balance is defined as intake minus excretion. A positive balance can be interpreted as retention in the body or metabolic degradation, whereas a negative balance can be interpreted as mobilization from the body or synthesis of the chemical. Data for all congeners except HpCDD, OCDD, and possibly OCDF, indicate that the cows were near steady-state because 0 was included within the range of balance values $\pm$ one standard deviation.

The excess excretion of HpCDD and OCDD can only be attributed to biosynthesis because the low concentrations of these congeners in milk at the start of dosing would indicate that body stores were too low to account for the level of excretion.[4] Excess excretion of these compounds in feces has been reported for humans,[6,7] and the formation of these congeners during composting and digestion of sewage sludge have also been reported.[8,9] Composting and sewage treatment are microbial processes. Since the gastro-intestinal tracts of animals are active microbial systems, we carried out *in vitro* fermentations with rumen microorganisms to determine if these organisms were the mechanism for formation of HpCDD and OCDD from precursors in the treated wood.

Table 1. Mass Balance of PCDDs and PCDFs in Dairy Cattle Administered Pentachlophenol-Treated Wood

Congener	Intake	Feces	Milk	Balance	Std. Dev.
	ug/day				
2,3,7,8-CDD	0.6	1.9	0.1	-1.4	0.9
1,2,3,7,8-CDD	5.2	3.2	1.0	1.0	0.5
1,2,3,4,7,8-CDD	13.0	17.8	1.6	-6.5	4.8
1,2,3,6,7,8-CDD	119	91	18.1	9.1	13.3
1,2,3,7,8,9-CDD	27.2	27.3	2.9	-3.1	7.6
1,2,3,4,7,8,9-CDD	3,430	6,500	105	-3,180	1,150
1,2,3,4,6,7,8,9-CDD	18,000	67,100	52	-49,200	10,040
2,3,7,8-CDF	2.3	5.4	0.0	-3.1	2.2
1,2,3,7,8-CDF	2.7	1.6	0.0	1.1	0.9
2,3,4,7,8-CDF	2.6	1.5	0.3	0.9	0.8
1,2,3,4,7,8-CDF	15.3	10.0	1.5	3.8	0.5
1,2,3,6,7,8-CDF	14.6	11.0	1.6	2.0	0.9
2,3,4,6,7,8-CDF	15.2	15.3	1.0	-1.1	2.4
1,2,3,7,8,9-CDF	1.0	1.3	0.0	-0.3	0.4
1,2,3,4,7,8,9-CDF	695	669	16.6	9.9	60.1
1,2,3,4,7,8,9-CDF	33.2	30.6	1.1	1.5	5.8
1,2,3,4,6,7,8,9-CDF	4,020	5,080	9.0	-1,070	407

Values are an average of four cows. Half the detection limit was used to calculate feed intake of 2,3,7,8-CDD, 1,2,3,7,8-CDD, and 1,2,3,7,8,9-CDF, which were below the detection limit. Balance = Intake (Feed +Wood) - Excretion (Milk + Feces).

Fermentation Characteristics. Several parameters were measured to determine if the level of PCP in the substrate had an adverse effect on microbial activity. Ruminal fatty acid production was not decreased by the addition of PCP-treated wood (Table 2). Acetate, iso-valerate and total volatile fatty acid production was higher in the samples containing the PCP-treated wood. Increased production of acetate led to an increased proportion of acetate relative to propionate and butyrate in the samples containing treated wood. Production of propionate, iso-butyrate, n-butyrate, and n-valerate were similar in both the control and treated samples.

Gas produced by fermentation is another indicator of microbial activity. Gas production was lower in the wood-containing samples than the control samples. Since, however, there was an increased production of total volatile fatty acids, reduced gas production does not indicate reduced microbial activity in the batch *in vitro* fermentations. Ammonia production during the

Table 2. Effect of 1% Added Pentachlorophenol-treated Wood on *in Vitro* Ruminal Fermentation

Product	Control	Wood	SEM	*P* <
Acetate, mmol/L	80.3	86.7	1.37	0.010
Propionate, mmol/L	20.0	20.9	.44	0.153
iso-Butyrate, mmol/L	2.3	2.0	.17	0.365
n-Butyrate, mmol/L	11.3	11.4	.27	0.916
iso-Valerate, mmol/L	2.7	2.4	.09	0.061
n-Valerate, mmol/L	2.0	2.0	.06	0.472
Total, mmol/L	118.5	125.4	2.12	0.050
Acetate, %	67.8	69.2	.30	0.011
Propionate, %	16.8	16.8	.20	1.000
n-Butyrate, %	9.6	9.0	.170	0.040
Acetate:Propionate	4.02	4.14	.051	0.134
Gas, ml/48 h	27.1	25.1	.33	0.013
Ammonia, mmol/L	71.4	75.0	2.67	0.367

incubation was similar between the control and treated samples indicating similar protein metabolism between the two types of samples.

The measured concentration of PCP in the substrate was 53 μg/g. This is equivalent to approximately 0.8 μg/ml in the 30 ml fermentation mixture. The effect of PCP on rumen microorganisms in comparable fermentation systems have been examined in two studies.[10,11] No adverse effects on fiber digestion were noted when the PCP concentrations in the fermentation mixture were 10 μg/ml, but fiber digestion was inhibited at 50 μg/ml in both studies. The lack of adverse effects in our study is consistent with the results of other studies. We conclude that the PCP in treated wood at the concentrations used in this study did not adversely affect *in vitro* fermentation, and that reliable inferences concerning PCDD synthesis can be drawn from these samples.

Effect of Fermentation on PCDD/F. The quantities of PCDD/F in the fermentation samples are presented in Table 3. The amounts of PCDDs in both the 0-h and 48-h samples do not differ from the amounts in the substrate and there is no evidence for the biosynthesis of HpCDD and OCDD. A number of PCDFs, most notably OCDF, were higher in amounts in 0-h and 48-h samples. The small number of replicates, however, do not permit one to draw definitive conclusions. Except for OCDF, the *in vivo* results (Table 1) do not support the conclusion the PCDFs are synthesized during ruminal fermentation.

The failure to demonstrate synthesis of HpCDD and OCDD in these fermentations with rumen organisms is not necessarily in conflict with the work on composting and sewage sludge digestion. Our system is completely anaerobic. In contrast, composting is aerobic and the

Table 3. Concentrations of PCDDs and PCDFs Due to the Fermentation of Pentachlorophenol-treated Wood by Rumen Microorganisms *in Vitro*

Congener	Substrate (N=1)	Background (N=1)	0 Hours (N=2)	48 Hours (N=3)
	pg/sample			
2,3,7,8-CDD	0.75	(1.1)	(1.5)	(1.5)
1,2,3,7,8-CDD	6.5	(1.0)	9.0	8.2
1,2,3,4,7,8-CDD	18.5	(0.7)	24	23
1,2,3,6,7,8-CDD	185	(0.7)	185	200
1,2,3,7,8,9-CDD	50	(0.7)	57	41
1,2,3,4,7,8,9-CDD	7,000	43	7,200	7,300
1,2,3,4,6,7,8,9-CDD	65,000	410	65,500	66,000
2,3,7,8-CDF	0.39	(1.1)	(1.4)	(0.9)
1,2,3,7,8-CDF	1.6	(1.3)	4.0	2.4
2,3,4,7,8-CDF	1.8	(2.0)	4.9	3.4
1,2,3,4,7,8-CDF	16.5	(0.6)	25	21
1,2,3,6,7,8-CDF	15.5	(0.7)	24	25
2,3,4,6,7,8-CDF	18.5	3.4	26	20
1,2,3,7,8,9-CDF	2.4	(0.4)	7.8	3.4
1,2,3,4,7,8,9-CDF	1650	14	1,750	1,700
1,2,3,4,7,8,9-CDF	48	(0.6)	52	52
1,2,3,4,6,7,8,9-CDF	6,000	48	7,350	7,300

Values in ( ) are half the detection limit for samples below the detection limit.

sewage sludge digestion system was described as semianaerobic.[8,9] Peroxidase enzymes can convert PCP to OCDD.[8] Possible mechanisms of HpCDD and OCDD formation could include (1) microbial action in the lower gut, (2) absorption of precursors and transformation in the liver with excretion in the bile, or (3) aerobic microbial action in the feces post excretion.

Conclusions

The following conclusions are drawn from this work.

1) Synthesis of HpCDD and OCDD occurred somewhere in cows following administration of PCP-treated wood.

2) Rumen microorganisms apparently are not the active agents in this synthesis based on the lack of conversion in an *in vitro* system.

3) The synthesis of HpCDD and OCDD in this and other biological systems provide an explanation for the relatively high concentrations of these congeners in environmental samples.

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