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Excretion of Hydroxylated Polychlorinated Biphenyl Metabolites in Cows' Milk

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Four lactating Holstein cows were each given a single oral dose of either 0.5 g 2,5,2′,5′-tetrachlorobiphenyl, 0.5 g 2,5,2′,4′,5′-pentachlorobiphenyl, 1.5 g Aroclor 1242, or 1.5 g Aroclor 1254 in gelatin capsules. Milk samples were collected twice daily at the am and pm milkings and were analyzed for polychlorinated biphenyl (PCB) metabolites and PCBs. Excretion of metabolites in the milk paralleled excretion of the parent PCBs, with maximum amounts eliminated 1 day after dosing. Less than 0.06% of the initial doses of the 2 single components appeared as metabolites in milk over a period of 10 days. Most of the metabolites occurred as conjugates. Only the 4-hydroxy metabolites were found in the milk of cows given single components. Milk samples from cows given Aroclor 1242 or Aroclor 1254 contained 10 and 4 monohydroxy metabolites, respectively. Using the same methodology, no monohydroxy metabolites were detected in whole milk samples from herds accidentally contaminated with a polybrominated biphenyl mixture.

Polychlorinated biphenyls (PCBs) (trade name Aroclor®) are industrial chemicals which occur as widespread contaminants in the global environment (1, 2). They have been found as residues in milk and dairy products in North America (3) and in Europe (4, 5). Their presence in milk has been traced, on occasion, to accidental contamination of cows' feed (3, 6, 7). The principal route of excretion of PCBs from the cow has been shown to be via the milk as opposed to excretion in the feces or urine (8).

Polybrominated biphenyls (PBBs) are industrial chemicals which are chemically similar to PCBs. PBB residues have also been found in milk (9) and meat (10) due to inadvertent feed contamination.

When milk samples containing PCB residues were analyzed by gas chromatography (GC), several workers observed a reduction of the lower chlorinated peaks (6, 11–14) as compared to the peaks found for commercial PCB products. The absence or reduction of these components relative to the higher chlorinated components suggested that they were either selectively metabolized, absorbed, or excreted by the cow. It has been shown recently that the cow does form a hydroxylated urinary metabolite of an Aroclor component, 4-chlorobiphenyl (15, 16), indicating that metabolism in the cow is an important means of elimination of PCBs. Other animals are also known to metabolize certain PCB components by hydroxylation (1, 17).

This exploratory study was conducted in order to determine the levels and nature of hydroxy-PCB metabolites in cows' milk after administration of a single PCB dose. Milk samples obtained from PBB-contaminated herds were examined for the presence of corresponding PBB metabolites using the same methodology.

Experimental

Dosing and Sampling

Four mature, lactating Holstein cows were each given a single dose of either 0.5 g 2,5,2′,5′-tetrachlorobiphenyl (TCB), 0.5 g 2,5,2′,4′,5′-pentachlorobiphenyl, 1.5 g Aroclor 1242, or 1.5 g Aroclor 1254 in gelatin capsules. Milk samples were taken from the 4 experimental animals and 2 untreated controls twice daily at 8 am and 3 pm, beginning 1 day before dosing and continuing for 11 days thereafter. At each milking 1 or 2 qt aliquots of milk from each cow were put into jars and kept frozen until analysis. Urethral catheters were installed in the cow given 2,5,2′,5′-TCB and a control cow for collection of urine for the first 2 days after dosing.

Milk containing PBBs was obtained from the Dairy Division of the Michigan Department of

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Agriculture (Lansing, MI). Three 1 pt aliquots were taken from bulk tanks known to hold milk from contaminated cows. Three other pt samples were taken directly from a PBB-contaminated cow.

Reagents and Apparatus

Except for toluene and ethanol, all solvents used were distilled in glass (Burdick and Jackson Laboratories, Inc., Muskegon, MI).

(a) *2,5,2',5'-TCB and 2,5,2',4',5'-pentachlorobiphenyl.*—Supplied by R. G. Webb (Environmental Protection Agency, Athens, GA) and Analabs, Inc. (North Haven, CT).

(b) *Aroclor 1242 and Aroclor 1254.*—Obtained from Food and Drug Administration (Washington, DC) standards collection.

(c) *Hydroxylated PCBs.*—Prepared by coupling 2,5-dichloroaniline or 2,4,5-trichloroaniline to 2,5-dichloroanisole as described previously (18). The isomers were identified by nuclear magnetic resonance analysis and GC-mass spectrometry (MS) (18). The synthetic hydroxy-PCBs were >99% pure as determined by GC (19), using flame and electron capture detection, and by thin layer chromatography (TLC) on fluorescent silica gel layers developed in toluene. Standard solutions were prepared in ethyl acetate for GC analyses.

(d) *Mass spectrometer.*—Finnigan Model 1015 C quadrupole, equipped with Finnigan 6000 data system.

(e) *Gas chromatograph.*—Varian Model 1700, equipped with 6' × 2 mm id 3% OV-101 on 80–100 mesh Chromosorb W (HP) column interfaced to mass spectrometer through Gohlke all-glass separator. Maintain 20 ml/min helium flow and 200°C column temperature.

Extraction

Milk.—Thaw milk samples overnight and then thoroughly blend at moderate speed. Extract PCB residues from milk by using the slightly modified procedure for high recoveries of fat as described by Beroza and Bowman (20). Mix 50 ml aliquot of milk with 50 ml absolute ethanol and 5 g NaCl. Extract this solution twice with 50 ml hexane-ethyl ether (1+1). Extract organic phase containing parent PCBs and hydroxy metabolites with 25 ml 1*N* NaOH. Backwash NaOH extract with 25 ml hexane-ethyl ether (1+1) and add upper layer to first organic extract. Concentrate an aliquot of this combined organic phase equivalent to 10 g milk under nitrogen and analyze for parent PCBs.

Acidify NaOH extract containing PCB metabolites as phenolates to pH 1 with concentrated HCl and then extract twice with 50 ml volumes of hexane-ethyl ether (1+1). Concentrate organic phase to 2 ml under nitrogen.

Acidify aqueous ethanol phase containing conjugated PCB metabolites to pH 1 with concentrated HCl and hydrolyze overnight at room temperature to liberate bound metabolites. Mix this solution with 200 ml hexane-ethyl ether (1+1) and filter. Draw off organic layer after separation and concentrate under nitrogen to 2 ml.

Analyze PBB and PBB metabolites in milk as described above for PCB and PCB metabolites.

Urine.—Extract 50 ml urine twice with 100 ml ethyl ether. Hydrolyze conjugated urinary metabolites as described above for milk and extract with 100 ml ethyl ether. Evaporate ether phase under nitrogen to 2 ml.

Cleanup

From extract containing PCBs, remove aliquot equivalent to 10 g milk in two 5 ml portions of petroleum ether. Clean up this sample on Florisil column (19) by elution with 200 ml petroleum ether. Concentrate eluate to 5.0 ml in Kuderna-Danish apparatus. Inject aliquot equivalent to ≤100 mg milk into gas chromatograph.

Streak milk and urine extracts for both free and conjugated (after hydrolysis) metabolite analysis on separate TLC plates (0.5 mm thick fluorescent silica gel layers). Develop plates with either methylene chloride or toluene for 7.5 cm. In methylene chloride system, 3-hydroxy- and 4-hydroxy-PCBs have an R_f between 0.53 and 0.80. In toluene system, 6-hydroxy-PCBs have an R_f between 0.76 and 0.93. Scrape adsorbent bands into glass wool-plugged disposable pipets which have been prerinsed with ethyl ether. Evaporate this solvent and quantitatively dissolve samples in 200 µl ethyl acetate. Inject aliquots equivalent to 1–5 g milk for GC analysis. Determine dihydro diols in urine as described by Gardner *et al.* (18).

Instrumental Analysis

Quantitate PBBs, PCBs, and PCB metabolites by GC, using tritium foil electron capture detection (19). Use different isothermal column temperatures (°C) in order to optimize GC of different groups of compounds: 200 for PCBs and their metabolites, 220 for some hydroxy-TCBs and hydroxypentachlorobiphenyls, and 260 for PBBs. Then perform MS analysis.

Results and Discussion

Recoveries

Fifty ng of each synthetic hydroxy-PCB was added to separate 50 ml aliquots of control milk. Samples were then extracted and cleaned up as

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described above. Recoveries of 4-hydroxy-2,5,2′,5′-TCB and 4-hydroxy-2,5,2′,4′,5′-pentachlorobiphenyl were 37 and 31%, respectively. These recoveries were used in correcting the quantitative data for the PCB metabolites in milk. Recoveries of the 3-hydroxy and 6-hydroxy isomers were in the 20–40% range. GC-MS evidence indicated that hydroxy-PCBs were partially oxidized to quinones during cleanup, and this may account for the low recoveries. Low recoveries have also been reported by other workers (21, 22). The cleanup described in this paper gave satisfactory separation of PCB metabolites from co-extracted fatty acids in milk with a minimum of sample workup.

Excretion of Metabolites

PCB metabolites were not found in control cows' milk or urine. Only one metabolite was found in the milk of each cow treated with either 2,5,2′,5′-TCB or 2,5,2′,4′,5′-pentachlorobiphenyl. The GC retention times of the metabolites found were identical to the respective 4-hydroxy compounds (Table 1). The urine of the cow given 2,5,2′,5′-TCB contained only 4-hydroxy-2,5,2′,5′-TCB, as determined by its previously reported GC retention time and infrared spectrum (18). Less than 15% of the metabolite was in the conjugated form in the urine. Although metabolite levels of 1.32 ppm (day 1) and 2.58 ppm (day 2) were measured in the urine, no other hydroxy metabolites, including dihydro diols, were found down to a screening level of 2 ppb. Safe *et al.* (16) observed that 4-chloro-4′-hydroxybiphenyl was the only urinary metabolite of 4-chlorobiphenyl in the cow. It may be concluded from the above data that the cow specifically metabolizes PCBs with unsubstituted 4-positions to the 4-hydroxy analogs.

Table 1 also shows the numbers and kinds of PCB metabolites found in milk. Ten hydroxy-PCB metabolites having 2–4 chlorine atoms/molecule were found in the milk of the cow fed Aroclor 1242. Four hydroxy-PCBs containing 4–5 chlorine atoms/molecule were found as metabolites of Aroclor 1254 in milk. The degree of chlorination of the parent PCBs (15, 23) was reflected in the amount of molecular chlorine in the metabolites. Apparently the lower chlorinated PCBs are metabolized more readily by

Table 1. Gas chromatographic and mass spectrometric data for synthetic and metabolic hydroxylated PCBs

Sample	R_A[a]	Spectrum No.[b]	Parent ion, m/e	No. of Cl atoms/molecule
Synthetic 4-hydroxy-2,5,2′,5′-TCB	1.54		306	4
Metabolite from cow given 2,5,2′,5′-TCB	1.54		306	4
Synthetic 4-hydroxy-2,5,2′,4′,5′-pentachlorobiphenyl	2.74		340	5
Metabolite from cow given 2,5,2′,4′,5′-pentachlorobiphenyl	2.74		340	5
Metabolites from cow given Aroclor 1242	[c]	23	238	2
		27	272	3
		38	238	2
		43	272	3
		53	272	3
		62	238	2
		80	306	4
		90	272	3
		93	272	3
		158	272	3
Metabolites from cow given Aroclor 1254	[c]	50	306	4
		118	340	5
		150	340	5
		203	340	5

[a] R_A = retention time relative to aldrin on 10% DC-200 column at 200°C. Nitrogen flow 120 ml/min.
[b] As obtained on 3% OV-101 column at 200°C. Helium flow 20 ml/min.
[c] Retention times of metabolites could not be matched with spectrum numbers due to slightly different chromatographic and detection conditions. R_A values on 10% OV-101 column at 200°C and nitrogen flow 120 ml/min for Aroclor 1242 metabolites were 0.56, 0.93, 1.16, 1.32, 1.62, and 1.73. R_A values for Aroclor 1254 metabolites under the latter conditions were 1.67, 2.54, and 3.16. R_A for 4-hydroxy-TCB was 1.68.

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the cow and some of these metabolites appeared in the milk. The presence of tetrachloro- and pentachlorobiphenyl metabolites supports the observation of Fries *et al.* that the tetrachloro- and some pentachlorobiphenyl components in Aroclor 1254 in milk appeared to have been readily metabolized by the cow (11). They also noted that other pentachlorobiphenyls were not as easily metabolized. Undoubtedly, the chlorine substitution pattern as well as the amount of molecular chlorine is important in determining rates of metabolism of PCBs (24, 25).

No monohydroxy metabolites of PBBs were detected in the PBB-contaminated milk, using the procedure to determine PCB metabolites. However, since no hydroxy-PBB compounds were available for testing, it is not certain that such metabolites would have been detected if present. PBB levels were as high as 900 ppb on a whole milk basis. The commercial PBB preparation suspected of contaminating the cows is composed of approximately 75% 2,4,5,2′,4′,5′-hexabromobiphenyl (C. Rappe, 1975, University of Umeå, Umeå, Sweden, personal communication). The lack of formation of PBB metabolites would be consistent with the currently held interpretation of PCB metabolism, viz., that highly halogenated biphenyl nuclei substituted in the 4-position are, at most, metabolized with difficulty in certain animal species (25–27).

Comparison of excretion rates of 4-hydroxy-2,5,2′,5′-TCB and its parent PCB in milk is shown in Fig. 1. Similar plots for parent and metabolites of 2,5,2′,4′,5′-pentachlorobiphenyl, Aroclor 1242, and Aroclor 1254 in milk have the same qualitative features. With few exceptions, the excretion of metabolites paralleled excretion of the parent PCBs, although at much lower levels, with a maximum excretion occurring 1 day after dosing. Higher excretion levels in the morning were probably due to observed higher volumes of milk in the morning. The amount of conjugated milk metabolites was higher than the amount of free metabolites in about 70% of the samples analyzed. Conjugates accounted for 54–82% of the total metabolites excreted. Figure 2 shows the comparative excretion in milk of free and conjugated metabolites of a component of Aroclor 1242. Variation in daily percentage of metabolites in the conjugated form was observed in plots of all metabolites.

Excretion of 4-hydroxy-2,5,2′,5′-TCB and 4-hydroxy-2,5,2′,4′,5′-pentachlorobiphenyl in milk could be quantitated by comparison with synthetic standards on the gas chromatograph. Less than 0.06% of the initial doses of 2,5,2′,5′-TCB or 2,5,2′,4′,5′-pentachlorobiphenyl appeared as metabolites in the milk over a 10-day collection period. By comparison, 10% of the 2,5,2′,5′-TCB dose was accounted for as urinary metabolite in only 2 days. Clearly, the major elimination route for this metabolite is through the urine and not the milk. On the other hand, the principal route of excretion of unmetabolized PCBs in the lactating cow was shown by Saschenbrecker *et al.* to be via the milk (8). The total amount of metabolites represented 21 and 18% of the total TCB and pentachlorobiphenyl components, respectively, present in the milk. The above excretion data reflect the cows' metabolism of one dose of a PCB component. Induction of metabolic enzymes after long term exposure to PCBs might possibly increase the levels of metabolites and the degree of conjugation. This phenomenon, however, has yet to be determined.

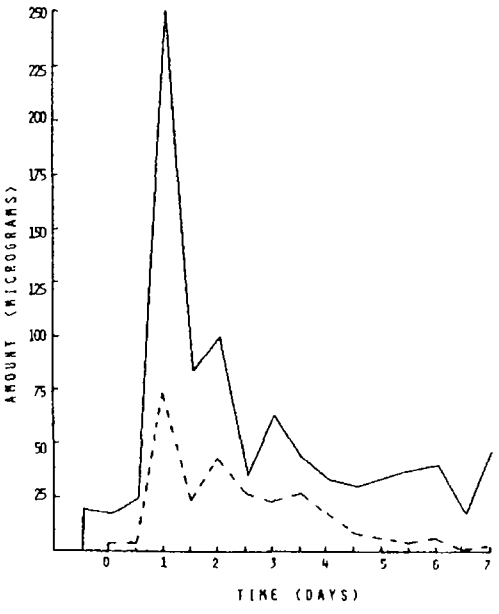

FIG. 1—Excretion of 2,5,2′,5′-TCB (——) and 4-hydroxy-2,5,2′,5′-TCB (– – –). Cow was given TCB on day 0. Data at half days represent afternoon samples. Amounts depicted are products of the ppb material found and the kg weight of the milk. The background level for TCB was about 10 ppb and is represented by the 25 μg level.

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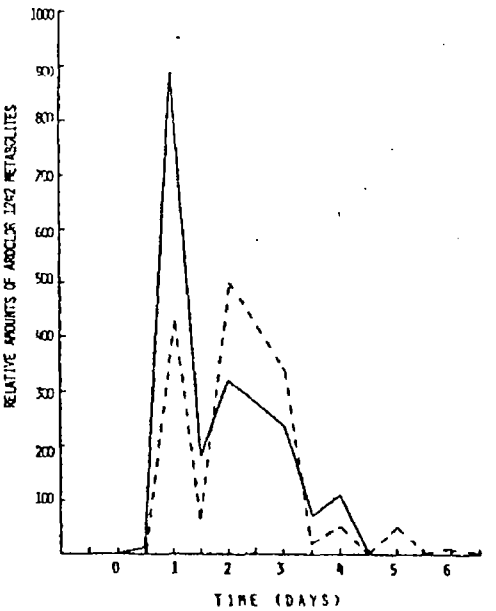

FIG. 2—Excretion of free (– – –) and conjugated (——) metabolites of Arocior 1242 having a retention time of 1.32 relative to aldrin. Cow was given Arocior 1242 on day 0. Data at half days represent afternoon samples.

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