Animal Bioavailability of a 3,4-Dichloroaniline–Lignin Metabolite Fraction from Wheat

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Treatment of intact wheat plants and excised shoot tissues with [U-^{14}C]-3,4-dichloroaniline led to 55–65% incorporation of the radioactivity into the "insoluble" residue. A sequential solubilization procedure revealed that ~85% of the ^{14}C-label was associated with the operationally defined lignin fraction. When the "insoluble" wheat metabolite residue was fed to rats and lambs, 11–20% of the bound radioactivity was released in soluble form. Refeeding the lamb fecal residue to rats released another ~7% of the bound radioactivity. A 3,4-dichloroaniline–lignin metabolite prepared enzymatically has previously been shown to be more extensively solubilized by rats (~66%). Mild acid hydrolysis under simulated stomach conditions (0.1 N HCl, 37 °C) resulted in a "burst" release of free 3,4-dichloroaniline (~30%) only from the previously used lignin metabolite. The presence of 4-hydroxybenzylamine linkages in only the lignin metabolites prepared enzymatically under mild conditions is proposed to explain the different degrees of solubilization in the digestive tract of animals. Animal bioavailability results can thus strongly depend on the methods used to prepare the "bound" metabolite fraction.

INTRODUCTION

Chlorinated anilines are formed as primary plant metabolites of acylanilide, phenylurea, and carbamate pesticides. The free chloroanilines are conjugated in plants, yielding soluble as well as "bound" (insoluble) conjugates (Still and Herrett, 1976). The latter are difficult to characterize, although cell wall lignin has been recognized to represent a major binding site [reviewed in Sandermann et al. (1983)]. Lignin is generally thought to be nondigestible in animals, so that chlorinated anilines bound to lignin would be nonbioavailable to an animal ingesting this kind of residue. Bound plant metabolites derived from a number of pesticides were in fact reported to remain largely undigested in animals (Harvey, 1983; Pillmoor and Roberts, 1985; Edwards and Hutson, 1986; Khan and Dupont, 1987; Akhtar, 1987). The ecotoxicological significance of a bound residue generally depends upon the bioavailability to an animal which ingests it and the potential release of low molecular weight xenobiotic fragments.

Definitions of bioavailability that differ in the assessment of the fecal fraction are in use in different laboratories. It seems therefore appropriate to briefly restate some basic definitions. According to Dorough (1976), when plant pesticidal bound residues "are administered orally to animals, the material excreted via the urine would be bioavailable; usually, that eliminated in the feces would be unavailable. That the fecal ^{14}C residues indeed were not absorbed from the gut could be confirmed by cannulating the bile duct and radioassaying the bile ...". Published bioavailability studies have indeed examined the bile fluid (Marshall and Dorough, 1977) or have, as an alternative, examined the soluble portion of fecal radioactivity (Bakke et al., 1972; Paulson et al., 1975; Khan et al., 1985, 1987, 1990).

In the absence of significant radioactivity in the bile fluid, high soluble fecal radioactivity could be formed by the intestinal microflora. In the presence of significant radioactivity in bile fluid, soluble fecal radioactivity could originate from biliary excretion, a process well documented for DDT and related compounds (Hayes, 1975). The existing two studies on animal bioavailability of bound chloroaniline metabolite fractions agree in describing high total fecal excretion rates. Biliary labeling was extremely low in one study, with feces not being tested for soluble radioactivity (Sutherland, 1976). In the second study, both bile fluid and the soluble portion of feces contained significant amounts of radioactivity. Bioavailability was in this case calculated as the sum of radioactivity excreted into bile, urine, and the soluble portion of feces and amounted to ~66% (Sandermann et al., 1990). A high degree of bioavailability with a significant contribution by soluble fecal radioactivity has recently also been determined in rats for the bound deltamethrin residues formed in wheat grains (Khan et al., 1990).

The high bioavailability previously described (Sandermann et al., 1990) was obtained with a well-defined chloroaniline–lignin model metabolite that can be made enzymatically in the laboratory (v.d. Trenck et al., 1981; Still et al., 1981; Arjmand and Sandermann, 1985). The present study was undertaken as a control experiment because it has remained an open question how closely the defined synthetic lignin polymer resembles the natural bound plant residues of chloroanilines. An in vivo chloroaniline metabolite fraction has now been prepared by treating wheat plants with [U-^{14}C]-3,4-dichloroaniline. The resulting wheat [U-^{14}C]-3,4-dichloroaniline residue was isolated according to standard methods and contained most of the 3,4-dichloroaniline as a lignin metabolite.

It will now be reported that this material was not

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extensively solubilized in the digestive tract of either the rat or the lamb. A chemical comparison of the previous enzymatically prepared and the present wheat lignin metabolite fractions of 3,4-dichloroaniline indicated that the two lignin preparations differed in the amount of 3,4-dichloroaniline bound by an acid-labile linkage. This chemical difference is proposed to explain the marked difference in bioavailability.

MATERIALS AND METHODS

Materials. [U-^{14}C]-3,4-Dichloroaniline with a specific activity of 33.58 μCi/mg was purchased from Pathfinder Laboratories. The reference *N*-glucoside of 3,4-dichloroaniline was prepared as described previously (Winkler and Sandermann, 1989). A wheat [U-^{14}C]-3,4-dichloroaniline–lignin metabolite fraction was prepared by pooling the thoroughly extracted insoluble wheat residues from the plant parts with the highest incorporation of ^{14}C (see below). The final dried bound residue fraction had a specific activity of 5.14 μCi/g. The enzymatically prepared [U-^{14}C]-3,4-dichloroaniline–lignin metabolite was the same material used in previous studies (Arjmand and Sandermann, 1985; Sandermann et al., 1990). Strong acid and base hydrolyses as well as solvolysis with 2-aminoethanol were performed by adopting standard literature procedures (Arjmand and Sandermann, 1985).

Treatment of Plants and Excised Tissues with [U-^{14}C]-3,4-Dichloroaniline. Field-grown winter wheat plants (*Triticum aestivum* L., var. Goetz) were 110 cm high and 9–10 months old. They had near-mature spikes. Semisterile wheat plants were produced as follows: Wheat seeds (*T. aestivum* L., var. Kolibri) were disinfected with hypochlorite and placed on autoclaved perlite with Hoagland nutrient solution. These plants were grown in small plastic growth chambers under ambient daylight conditions. Six-week-old plants were 40 cm high and were used for root uptake experiments. Treatment with [U-^{14}C]-3,4-dichloroaniline (2–5 μCi/plant; specific activity 5.44 mCi/mmol) was done by the following three methods that led to similar incorporation rates into the bound residue fraction.

1. Injection Method. [U-^{14}C]-3,4-Dichloroaniline (2 μCi in 4 μL of methanol) was injected by syringe into the upper part of the first or (preferably) second stem internode from the top of the field-grown wheat plant. This was followed by 20–30 days of further cultivation under ambient daylight conditions and on the original soil substrate, with regular watering. Autoradiography showed that 90% of the radioactivity was associated with the internode used for injection and the adjacent 10-cm apical stem section. These parts of the wheat plants (about 8% of plant fresh weight) were excised and used for workup. The weight of the spike amounted to about 48% of the total fresh plant weight.

2. Infiltration Method. The field-grown wheat plants were cut 10 cm below the second node, and the upper section of the plant parts (about 70 cm length) were maintained under ambient daylight conditions in test tubes containing 5 mL of water each. Treatment was by addition of 5 μCi of [U-^{14}C]-3,4-dichloroaniline in 10 μL of methanol per 5 mL of water. After the application, ca. 10 mL of water was added to maintain a 15-cm level in the test tubes. Water was consumed at a rate of 2–5 mL/day per plant. Treatment was continued for 20–30 days. Autoradiography indicated that most of the radioactivity was associated with the immersed tissues and the next 5-cm stem section. This portion of the tissue was excised, rinsed with water, and used for workup. Dichloromethane/methanol/water solubilized a low amount (about 1%) of the applied radioactivity from the spikes and less (about 0.5%) from the leaves.

3. Root Uptake Method. The hydroponic wheat plants, after removal of perlite, were transferred into test tubes (16-mm diameter, 16-cm height). These were filled with water to a level of ca. 10 cm, so that the whole root was immersed. [U-^{14}C]-3,4-Dichloroaniline (5 μCi in 10 μL of methanol) was added to the water phase (ca. 15 mL), and root uptake was allowed to proceed for 4–31 days under ambient daylight conditions.

The plants were periodically resupplied with water which was taken up at a rate of 2–5 mL/day per plant. Autoradiography and extraction showed that after 4 days, most of the applied radioactivity had been taken up into the plant and was partitioned between roots, stems, and leaves. Most of the radioactivity was present in the stems.

Workup Procedure. After all three feeding procedures, the plant sample was first rinsed with water, then cut by use of scissors, and ground in liquid nitrogen to a fine powder using a Retsch shaker with a 10-mL Teflon cell and a Teflon-coated steel ball. Large-scale homogenization was then performed using a Polytron instrument (Kinematica) with a 2-L glass container with cooling by liquid nitrogen. The powdered plant material used for cell wall characterization was thoroughly extracted (at least 10 times) with methanol/dichloromethane (2:1 v/v) and dichloromethane/methanol/water (1:2:0.8 v/v/v).

Dosing Procedure for Rats. Ground rat feed (18.5 g) and wheat [^{14}C]dichloroaniline–lignin metabolite (0.8 g) were mixed using a mortar and pestle. Several samples were assayed for ^{14}C to ensure that homogeneity had been achieved. Each rat (200–300-g male Sprague-Dawley) was offered an accurately weighed amount (approximately 3 g, 0.76–0.9 μCi) of the mixture in a stainless feed cup. The rats had been trained to consume a meal relatively rapidly, and the dose was completely consumed within 30 min. Less than 0.1% of the dose could be recovered from the feed cups. Bile cannulations [as described by Struble et al. (1983)] were performed 24 h before dosing. The rats were held in restraining cages (Bollman, 1948) from 24 h before dosing until sacrifice. Feces and urine were collected separately from time of dosing until sacrifice.

After dosing, the rats were fed two 7-g meals a day and water ad libitum. All rats were sacrificed 72 h after dosing, by which time most of the ^{14}C-labeled material had been excreted. Samples of urine and bile were assayed directly by liquid scintillation counting. Feces and tissues were homogenized and freeze-dried; a sample was combusted, and the resulting $^{14}CO_2$ was trapped for assay by liquid scintillation counting (Packard Model 306 oxidizer, Packard Instrument Co., 2200 Warrenville Rd., Downers Grove, IL 60515).

Dosing Procedure for Sheep. A portion of the wheat [U-^{14}C]-3,4-dichloroaniline–lignin metabolite was combusted, and specific radioactivity was redetermined. A known amount was placed into gelatin capsules, which were given orally to castrated male sheep, weighing 16–22 kg. The sheep were kept in metabolism cages and fitted with a urine collection apparatus (Paulson and Cotrell, 1984). Urine and feces were collected separately from time of dosing until sacrifice. A catheter was installed in the bile duct of one sheep 96 h before dosing. A 2.5 mM solution of ox bile salts in Ringers solution was infused at approximately the same rate as bile flow and bile was collected. Upon dosing, the sheep bile previously collected was then used for the infusions until sacrifice. The sheep were sacrificed 96 h after dosing. The handling of urine, bile, feces, and tissues was identical to the procedures described previously for the rat experiments.

Extraction of Soluble Metabolites from Feces. Feces samples were freeze-dried, suspended in a solvent mixture of chloroform/methanol/water (5:10:4), and stirred for several hours. The suspension was filtered to give a liquid phase of soluble radioactivity and a solid pellet of insoluble radioactivity. The solid pellet was ground and re-extracted with the same solvent mixture to make sure that all soluble radioactivity was removed by the initial extraction. The final pellets were dried and in some cases combusted for determination of radioactivity. In all cases, the final dry weight averaged 83% of the initial feces dry weight. A mixture of chloroform/methanol/0.1 N HCl (5:10:4) was used for some feces extractions but did not lead to greater extraction rates than either the first or second extraction with the neutral solvent mixture. Sonication of sheep feces in place of or in addition to stirring also failed to increase extraction efficiency.

Dosing Procedure for Refeeding Experiment. A portion of the freeze-dried feces from lamb 2 was extracted with chloroform/methanol/water (5:10:4 v/v/v) until no more radioactivity could be removed. The remaining solid was dried, ground to apparent homogeneity, and radioassayed. The specific radioactivity was 1.114×10^5 dpm/g. Feces of untreated sheep was extracted accordingly. Rats were trained to eat a 5-g meal

containing approximately 20% of the extracted nonradioactive sheep feces within 30 min. Six rats were given a 0.05-μCi dose of the extracted radioactive feces from lamb 2 as a 20% mixture with rat feed. All rats consumed the entire dose within 45 min. Feces and urine were collected separately from time of dosing until sacrifice. After dosing, the rats were fed two 8-g meals a day and water ad libitum. All rats were sacrificed 72 h after dosing, and the samples and tissues were handled as in previous experiments.

Chromatographic Procedures. Thin-layer chromatography (TLC) was carried out on precoated silica gel 150 A plates (Whatman LK5F) using the following solvent systems (ratios volume): A, methylene chloride/methanol 9:1, B, *n*-hexane/toluene/acetone 7:3:1; C, ethyl acetate/2-propanol/water 6:2:1; D, ethyl acetate/acetic acid/water 120:10:1; E, acetonitrile/water/concentrated ammonia 7:2:1. R_f values for 3,4-dichloroaniline and D-glucose were 0.2 and 0 (solvent system B) and 0.8 and 0.1 (solvent system C), respectively. Bile samples were not studied due to minimal radioactivity contained therein. The urine samples were chromatographed through C_{18} Sep-Pak cartridges (Waters Associates, Inc.) using water, followed by methanol as eluents.

The methanol fractions were concentrated and reapplied to C_{18} Sep-Pak cartridges using water followed by 5% aqueous methanol as eluents. The 5% methanol in water fraction contained approximately 80% of the applied radioactivity and was concentrated and redissolved in water. The aqueous solution was adjusted to pH 8 by addition of solid sodium bicarbonate, and the solution was extracted several times with ethyl acetate. The ethyl acetate fractions were concentrated and analyzed by TLC using solvent system A.

Cleanup Procedures. Water was added to the organic extract containing the soluble radioactivity from feces to induce the formation of a two-phase system. Since the aqueous phase typically contained only about 5% of the total radioactivity, the organic phases were collected, combined, and concentrated. Hexane extraction of the organic phase did not remove significant radioactivity. The organic phases were concentrated in the presence of a small amount of silica gel, and the resulting powder was applied to a column of silica gel. Stepwise elution was performed beginning with 100% hexane to 100% ethyl acetate, followed by stepwise elution from pure ethyl acetate to 100% methanol. About 80% of the radioactivity was eluted with 10–30% methanol in ethyl acetate. The missing radioactivity appeared to be bound to the silica gel and was not removed with methanol. The radioactive solutions were combined, concentrated, and analyzed by TLC using solvent system C.

Mild Acid Hydrolysis. The enzymatically prepared 3,4-dichloroaniline lignin metabolite (in 10 μL of DMF, 14 800 dpm) or the powdered wheat 3,4-dichloroaniline–lignin metabolite (~1 mg of dry weight, 11 000 dpm), and 3 μL of nonradioactive, 3,4-dichloroaniline (10 mg/mL methanol) were added to 250 μL of 0.1 N HCl each and incubated in a shaking water bath at 37 °C for various time periods. The samples were cooled on ice and made slightly alkaline by addition of 290 μL of 0.1 N NaOH. Each sample was brought to a volume of 3 mL by addition of water and then extracted three times with 1-mL portions of ethyl acetate. Aliquots from the combined ethyl acetate phases were taken for liquid scintillation counting and for TLC analysis in solvent systems B and C. Radioactive material migrated as a single peak with or near the added 3,4-dichloroaniline which was visualized under UV light.

In control experiments, the above procedure was modified by carrying out the hydrolysis in 1,4-dioxane/water (8:2 v/v) containing 0.1 N HCl.

RESULTS

Metabolism of [U-^{14}C]-3,4-Dichloroaniline by Wheat Plants. In each of the three different plant treatment methods described under Materials and Methods, 55–65% of the initial radioactivity of [U-^{14}C]-3,4-dichloroaniline was found to become associated with the "insoluble" wheat residue. The aqueous dichloromethane–methanol extracts were examined by TLC (solvent system E). In each case, one main peak (≥70% of applied radioactivity) appeared at the R_f value of authentic *N*-glucoside (R_f 0.65). In addition, two or three unidentified peaks were present. This was also true for the minor translocation sites of radioactivity (spikes, leaves). The *N*-glucoside was purified by HPLC (three runs) on RP-8 and RP-18 columns. It could be firmly identified by TLC and HPLC before and after acetylation by comparison with the authentic reference compound [cf. Winkler and Sandermann (1989)]. In EI mass spectroscopy of the acetylated metabolite, the parent ion appeared at *m/z* 491, with its characteristic companion ion at *m/z* 493. Further typical fragments at *m/z* 331, 270, 245, 169, and 109 were all present in the same proportion as in the acetylated reference *N*-glucoside of 3,4-dichloroaniline.

Table I. Distribution of Radioactivity in the Various Fractions of the Sequential Solubilization Procedure of Langebartels and Harms (1985)[a]

treatment with	% ^{14}C solubilized	treatment with	% ^{14}C solubilized
organic solvent	1.2	dioxane/2 N HCl, 70 °C	84.9
0.1 M potassium phosphate	1.9	1 N KOH	1.8
α-amylase	1.1	81% (w/w) aqueous H_2SO_4	0.7
Pronase E	1.0	final dark residue	1.5
50 mM EGTA, pH 4.5	4.3	losses to filters	1.6
		total	100.0

[a] The original step utilizing neutral dioxane/water (9:1 v/v) or dimethyl sulfoxide was omitted because pilot experiments had shown that this time-consuming treatment released only a little radioactivity. A wheat cell wall fraction (0.4 g) prepared by the infiltration method was employed. Radioactivity was determined as previously described (Sandermann et al., 1990).

Characterization of the Insoluble Wheat Residue. An aliquot (0.4 g) of the ground and extracted residue fraction obtained by the infiltration method was further characterized by the sequential solubilization procedure of Langebartels and Harms (1985). The results are shown in Table I. About 85% of the radioactivity was present in the operationally defined lignin fraction.

To obtain sufficient radioactive material for animal feeding studies, the most highly labeled residue samples from the present three plant treatment methods were combined. The pooled material consisted of 12.55 g of powdered and extracted insoluble wheat residue with a total amount of radioactivity of 64.5 μCi. This material was taken for the animal treatment experiments. Silicates are often found in field-grown plant cell walls and can strongly influence animal digestibility. The combined wheat cell wall fraction contained 10 μg of silicium/g of dry weight (kindly determined by Doz. Dr. P. Schramel, GSF-Institut für Ökologische Chemie, Neuherberg).

Determination of Bioavailability. Male albino rats and male sheep were used. Prior to treatment, the polymeric nature of the 3,4-dichloroaniline–lignin metabolite was confirmed by TLC with solvent system B. Polymeric material remained at the origin, whereas any free chloroanilines or other degradation species would have separated. Radioactivity was located only at the origin.

The distribution of radioactivity upon sacrifice is shown in Table II. Bioavailability calculated as the sum of radioactivity excreted into bile, urine, and the soluble portion of the feces (see Introduction) averaged 12.1% for five rats and 20.0% for the two sheep. These data suggest that the digestive tract of a ruminant and a monogastric animal may slightly differ in their ability to solubilize the radioactivity in the wheat [U-^{14}C]-3,4-dichloroaniline–

Table II. Distribution of Radioactivity after Orally Dosing Animals with the Wheat [U-^{14}C]-3,4-Dichloroaniline–Lignin Metabolite[a]

	% of dose						
	rat					lamb	
	1	2	3	4	5	1	2
feces-bound	66.0	89.9	86.3	87.5	89.8	82.4	83.6
feces-soluble	4.3	5.0	6.6	6.2	5.6	9.8	9.4
urine	6.3	5.5	4.0	4.3	5.8	7.9	12.1
bile	3.8	3.0				0.8	
GI tract	1.1	1.2	<0.1	<0.1	<0.1	0.9	0.5
carcass	0.5	0.4	0.2	0.2	0.3	1.4	1.0
total recovery	82.0	104.9	97.1	98.3	101.5	103.2	106.6
bioavailable	14.4	13.5	10.6	10.5	11.4	18.5	21.5

[a] Interval from dosing to sacrifice was 72 h for rats, 96 h for sheep.

Table III. Distribution of Radioactivity after Feeding Rats Extracted Feces from Lamb 2[a]

	% of dose		% of dose
feces-bound	90.7 ● 6.9	GI tract	<0.1
feces-soluble	4.3 ± 0.8	carcass	<0.1
urine	2.9 ± 0.8		
total recovery	97.8 ± 7.0		
bioavailable	7.1 ± 0.4		

[a] Interval from dosing to sacrifice was 72 h. Average values ± standard deviation are given (n = 6).

lignin metabolite. The urinary and fecal excretion rates were similar to those reported for the synthetic [U-^{14}C]-3,4-dichloroaniline–lignin metabolite when fed to rats (Sandermann et al., 1990). However, in that study, total bioavailability of the radioactivity amounted to 66%, due primarily to soluble radioactivity in feces.

This high degree of soluble fecal radioactivity was not found in the present system. Excretion of radioactivity in the bile fluid was also noticeably lower in the present study. In the previous study, it had amounted to greater than 10% of the radioactivity (Sandermann et al., 1990). Although considerable effort was expended (extractions, HPLC, TLC, GC), the urinary or soluble fecal radioactivity could not be isolated as discrete fractions and characterized: either the radioactivity exists as numerous different compounds or the systems utilized were not capable of isolating the labeled compounds that were present.

Results of Refeeding. Table III shows the distribution of radioactivity after rats were treated with extracted feces from a sheep dosed orally with wheat [U-^{14}C]-3,4-dichloroaniline–lignin metabolite. Bioavailability averaged 7.1 ± 0.4% for the six rats. This percentage was similar to that of three conventional rats (10.8 ± 0.5%) dosed orally with the wheat 3,4-dichloroaniline–lignin metabolite. This indicates that the ^{14}C in the wheat 3,4-dichloroaniline–lignin metabolite and the insoluble ^{14}C in the feces of sheep dosed with the wheat [^{14}C]-3,4-dichloroaniline–lignin metabolite have similar bioavailabilities in the rat digestive tract.

Chemical Characterization of 3,4-Dichloroaniline–Lignin Metabolites. In view of the considerable difference in bioavailability between the previously studied enzymatically prepared chloroaniline–lignin metabolites (Sandermann et al., 1990) and the present wheat residue, both types of lignin compounds were compared with regard to their chemical properties according to the previous methods (Arjmand and Sandermann, 1985). Both lignin metabolites were sensitive to strong acid and base hydrolysis and were most efficiently cleaved by solvolysis with 2-aminoethanol.

Cleavage rates in 70% H_2SO_4, 50% NaOH, and 2-aminoethanol were 92, 32, and 84%, respectively, for the enzymatically prepared lignin metabolite and 24, 23, and 90%, respectively, for the wheat lignin metabolite. The released radioactivity was extracted into ethyl acetate and migrated upon TLC as a single broad peak together with or close to the reference compound, 3,4-dichloroaniline, indicating similarity to the reference, possibly with slight modification (e.g., hydroxylation). No further attempt was made to identify hydrolysis products. The results obtained sufficed to exclude the possibility that labeling of the wheat lignin metabolites had occurred by refixation of $^{14}CO_2$ [cf. Sandermann et al. (1983)].

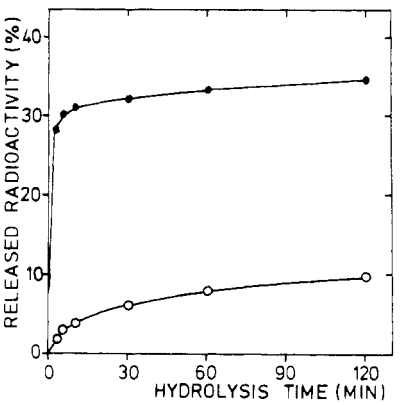

Figure 1. Mild acid hydrolysis (0.1 N HCl, 37 °C) of the [U-^{14}C]-3,4-dichloroaniline–lignin metabolite prepared enzymatically (Arjmand and Sandermann, 1985; ●) and of the [U-^{14}C]-3,4-dichloroaniline–lignin metabolite prepared with wheat plants (○). The percent release of radioactivity is plotted vs time of acid hydrolysis.

Because of the high degree of bioavailability of the enzymatically prepared 3,4-dichloroaniline–lignin metabolite, mild acid treatment under simulated stomach conditions (0.1 N HCl, 37 °C) was performed with both lignin metabolites. The time dependence of hydrolysis is shown in Figure 1. The enzymatically prepared lignin metabolite of 3,4-dichloroaniline (and of 4-chloroaniline; data not shown) was sensitive to mild acid. After an initial burst of ^{14}C release (~30%), there was a linear release phase. A total of ~35% of the initial radioactivity was released after 2 h. The cleavage products migrated upon TLC as a defined peak together with or close to the 3,4-dichloroaniline reference compound. The wheat lignin metabolite fraction showed no burst release and gave a linear release of only ~10% total hydrolysis after 2 h. The released radioactive material again chromatographed upon TLC close to the 3,4-dichloroaniline reference compound.

The percentages of hydrolysis of Figure 1 may be compared to the bioavailability rates reported previously (Sandermann et al., 1990) and in the present Table II (68 and 15%, respectively). However, similarities could be due to a trivial factor, namely the different physical states of the two lignin samples studied.

Accessibility to acid can be expected to be different for a lignin dissolved in organic solvent, as compared to lignin associated with an insoluble cell wall powder.

The acid hydrolysis experiments of Figure 1 were therefore repeated in 80% (v/v) aqueous 1,4-dioxane, which is known to be an effective lignin solubilizing agent and which was thought to facilitate the penetration of HCl into the cell wall matrix. In the presence of 80% (v/v) 1,4-dioxane, a hydrolysis curve identical to that shown in Figure 1 was obtained for the enzymatically prepared lignin metabolite. The wheat lignin metabolite released twice

as much (~20%) radioactivity as before, but still no burst release occurred.

Treatment of the lignin samples with water rather than 0.1 N HCl released ~15% of the bound radioactivity from the enzymatically prepared lignin metabolite, whereas the wheat lignin metabolite was water-stable. The partial water sensitivity of the enzymatically prepared lignin sample was also observed in TLC using solvent system C. In contrast to the complete immobility in the nonaqueous solvent system B [cf. Sandermann et al. (1990)], 15% of the applied radioactivity was released and cochromatographed with the 3,4-dichloroaniline reference compound.

DISCUSSION

Preparation of a [U-^{14}C]-3,4-Dichloroaniline–Lignin Metabolite Fraction. The present three plant treatment methods led to incorporation of [U-^{14}C]-3,4-dichloroaniline into the bound "residue" fraction in a yield of ~60%. A previous study in which [U-^{14}C]-3,4-dichloroaniline was applied to hydroponic wheat plants had led to 24% incorporation of the applied radioactivity into the insoluble residue (Arjmand and Sandermann, 1986). In the case of wheat cell cultures, incorporation rates of 65% for 4-chloroaniline and 31% for 3,4-dichloroaniline have been obtained (Harms and Langebartels, 1986; Langebartels et al., 1986; Winkler and Sandermann, 1989). 4-Chloroaniline was incorporated into the bound residue fraction in yields of 7.3 and 42% when cell suspension cultures of tomato and maize, respectively, were studied (Pogàny et al., 1990).

The incorporation of chlorinated anilines into cell wall lignin had in the previous study with hydroponic wheat plants (Arjmand and Sandermann, 1986) been shown by the following additional methods: lignin extraction according to the method of Björkman, UV spectroscopy, gel permeation chromatography on Sephadex LH-60, and three standard degradation procedures. The latter procedures had shown that true xenobiotic incorporation of the chloroanilines rather than labeling via $^{14}CO_2$ had occurred. The same finding has been made for the present metabolite material from wheat (cf. Table III).

Lignin was identified as a major incorporation site of one of the employed fractions by the sequential solubilization procedure of Langebartels and Harms (1985) (cf. Table I; ~85% incorporation into the operationally defined lignin fraction). This percentage compares to the ~60% reported for rice roots (Sutherland, 1976; Still et al., 1981). In the case of the bound residue formed in wheat cell cultures from 4-chloroaniline, the same fractionation procedure had shown 48% incorporation into pectins and only 27% incorporation into lignin (Langebartels et al., 1986). Similar distributions of radioactivity were determined for the bound residues formed from 4-chloroaniline in tomato and maize cell cultures (Pogàny et al., 1990). The lower lignin incorporation rate in cell cultures may reflect the chemical difference between the generally pectin-rich cell walls from cultured plant cells and the high lignin content of straw-forming wheat plants.

Animal Bioavailability. Bioavailability is defined here as the sum of urinary, biliary, and soluble fecal radioactivity (see Introduction). The bioavailability of the wheat 3,4-dichloroaniline–lignin metabolite was ~12% in rats and ~20% in lambs. Another 7% of the bound radioactivity was released when the excreted lamb fecal material was fed to rats. The latter finding is consistent with the extended linear release phase in the model experiments of Figure 1. The bioavailability rates were much lower than the bioavailability of ~66% observed previously in rats for chloroaniline–lignin metabolites prepared enzymatically (Sandermann et al., 1990). The present low degree of bioavailability is, however, close to that reported in the only comparable previous study, where bound 3,4-dichloroaniline residues from rice plants had been fed to rats (Sutherland, 1976).

Relationship between Chemical Structure and Animal Bioavailability. Chemical degradation, in particular with 2-aminoethanol, established the truly xenobiotic nature of the two lignin metabolite preparations used for the animal digestibility studies (Table III). It was furthermore discovered that mild acid hydrolysis under simulated stomach conditions partially reproduced animal bioavailability (Figure 1). In particular, it was remarkable that ~30% of the bound 3,4-dichloroaniline of the artificial sample was released almost instantaneously in a burst reaction. Release from the wheat lignin metabolite fraction occurred much more slowly and in a linear time dependence. Therefore, two main populations of bound 3,4-dichloroaniline appeared to exist. The rapidly released portion of the enzymatically prepared lignin metabolite was also partially released in water without added HCl.

Acid-labile lignin-related model adducts have been reported previously in the literature. The first report on a labile linkage in lignin seems to be that by Freudenberg (1968). This author was able to bind carbohydrates to the α-carbon of a lignin model compound and to characterize this linkage as being sensitive to mild acid (Freudenberg, 1968). The α-carbon in lignin side chains is generally known to be by far the most reactive lignin linkage in hydrolysis and solvolysis reactions (Allan, 1971; Wallis, 1971; Azuma, 1989; Monties, 1989). It is further known (Freudenberg, 1968; Allan, 1971; Wallis, 1971) that α-substituents in lignin subunits and model compounds containing a free hydroxyl group in the para position to the α-carbon are much more labile than those in lignin subunits in which the *p*-hydroxyl group is derivatized.

The burst release from the artificial sample is therefore most likely due to 3,4-dichloroaniline bound as a 4-hydroxybenzylamine substituent. This partial structure (Figure 2) was identified originally as a major incorporation mechanism for chlorinated anilines (v.d. Trenck et al., 1981; Still et al., 1981). On the basis of weight ratios and NMR spectroscopy, much of the lignin incorporation was due to benzylamine linkages to the α-carbon (v.d. Trenck et al., 1981; Sandermann et al., 1983). The lack of the burst release of the 3,4-dichloroaniline–wheat lignin metabolite was on the basis of the control experiment with 1,4-dioxane, not due to a physical barrier. The present data cannot differentiate between the following two possible explanations: (1) The labile 4-hydroxybenzylamine linkage is truly absent or present only to a minor degree (<2%) in native wheat cell walls. It may be an artifact of in vitro lignin formation or disappear during cell wall aging. (2) The labile 4-hydroxybenzylamine linkage is broken during cell wall isolation, even though mild methods were employed. This could, for example, occur by disrupting the native structure of the hydrophobic lignin phase. At this time, one can only conclude that the animal bioavailability results obtained strongly depend on the methods used for preparation of the 3,4-dichloroaniline–lignin metabolites.

Simmons et al. (1989) have carried out thorough studies on the peroxidase-catalyzed co-oligomerization of 4-chloroaniline and guaiacol (2-methoxyphenol). The latter can be regarded as being derived from coniferyl alcohol by loss of the propanoid side chain. Formation of a 4-hy-

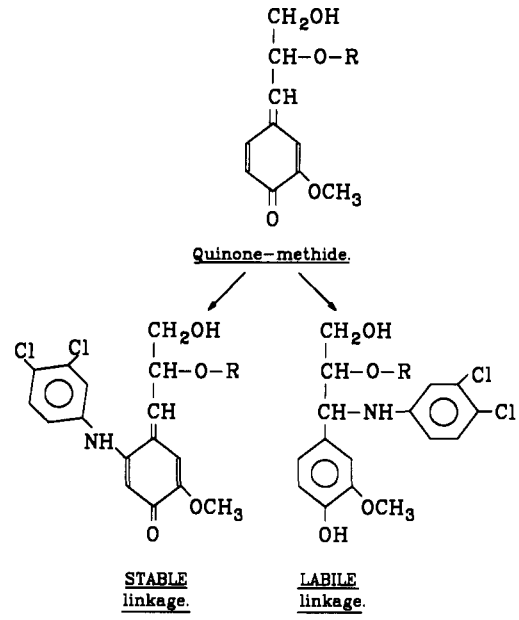

Figure 2. Examples for acid-labile (right) and acid-stable (left) linkages of 3,4-dichloroaniline to a lignin subunit. The right-hand structure was taken from v.d. Trenck (1981) and Still et al. (1981). The left-hand structure is likely to further react and was adopted from the chemical structures elucidated by Simmons et al. (1989) for guaiacol/4-chloroaniline adducts.

droxybenzylamine linkage is therefore impossible. Simmons et al. (1989) structurally elucidated several linkage types between 4-chloroaniline and guaiacol-derived oligomers. These linkages were stable to mild acid (pH 2.5). One of the elucidated linkage types has been adopted in Figure 2. Similar rather stable quinoid adducts of chlorinated anilines have been described by You et al. (1982). Figure 2 thus summarizes the limited present literature evidence for the existence of acid-labile and acid-stable linkages between chloroanilines and lignin or humus-type substances. These structures are still largely hypothetical, and there is a need for thorough chemical studies. It should furthermore be noted that Simmons et al. (1989) as well as much previous literature indicate that plant peroxidases will also catalyze transformation reactions of 4-hydroxycinnamyl alcohols and guaiacol as well as chloroanilines and that the secondary products formed will also readily copolymerize. These more complex binding processes have not been taken into account in Figure 2.

Conclusions. It is concluded that the partial structure previously derived for the enzymatically prepared lignin metabolites of chloroanilines (Figure 2, right; Still et al., 1981; v.d. Trenck et al., 1981; Sandermann et al., 1983) represents a labile binding site. Indications for a labile linkage between lignin and a xenobiotic are apparently reported here for the first time. Labile linkages may to a certain degree be protected in native plant cell walls by being localized within the hydrophobic microenvironment of the undisturbed lignin phase. This type of linkage could be involved in normal plant cell wall extension growth which requires reversible cross-linkages, although recent reviews on cell wall cross-links (Fry, 1986; Roberts, 1990) fail to mention labile lignin cross-linkages.

Labile linkages of natural lignin substituents are likewise expected to be broken under stomach conditions, releasing quinone methides that could undergo secondary addition reactions in the intestinal tract.

The present study illustrates the potential usefulness of one of the criteria (simulation of stomach pH) recently proposed in a scheme for risk assessment of bound xenobiotic metabolites in animals (Lu et al., 1990). This criterion has previously been shown to apply to the *N*-glucoside conjugates of chlorinated anilines (Sandermann, 1987; Winkler and Sandermann, 1989). The development of simple chemical methods to characterize soluble and insoluble pesticidal plant metabolites could give first indications for bioavailability and could thereby reduce the number of required animal experiments.

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