is stored in vacuoles of juice vesicle cells. The proteins, however, are synthesized at the site of ribosomes, which are located in the cell cytoplasm at a pH close to 5.5. The presence of many neutral amino acids (Table II) would tend to keep many of these proteins insoluble at this pH, due to isoelectric effects. When the cloud proteins are introduced to the pH 2.5 environment, after rupture of the citric acid vacuoles during processing, the proteins could remain insoluble due to a free energy barrier. The chaotropic effect of the urea, however, could allow the proteins to overcome this barrier and to become soluble. Considering the heterogeneity of the cloud proteins, when the urea is removed via dialysis it is unlikely that all of these proteins would revert to their insoluble state, due to many irreversible conformational changes that would be expected to occur.

We conclude that lemon cloud protein is a complex, heterogeneous material comprising approximately one third of the total cloud weight. It is probably derived primarily from the endocarp of the fruit, but it may also contain some material from the albedo. It appears to be composed, in part, of inherently insoluble protein which probably is derived from cellular organelles and lipid membranes. Some protein complexes with another fruit constituent could also play a role.

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Registry No. Pectin esterase, 9025-98-3.

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Bioavailability in Rats of Bound ^{14}C Residues from Corn Plants Treated with [^{14}C]Atrazine

Shahamat U. Khan,* Sam Kacew, and Stephen J. Molnar

Corn plants grown to the silage stage were treated with ^{14}C ring-labeled and unlabeled atrazine. The aerial portion of the plants was exhaustively extracted with solvents and the extracted material containing bound ^{14}C residues was fed to rats. For comparison extracted material from control plants fortified with [^{14}C]atrazine was also fed to rats. After 4 days, 88% of the dose was excreted in the feces and 10% in the urine from rats fed plant material containing bound ^{14}C residues. In contrast, only 32% of the dose was eliminated in the feces while 60% was voided via the urine when the corn material fortified with [^{14}C]atrazine was fed to rats. Most of the ^{14}C residues in feces from rats fed bound diet remained nonextractable and their amounts and nature were similar to those in the corn material. Atrazine added to the corn material before feeding was metabolized effectively when consumed by rats. The data demonstrated that bound residues in corn plant treated with atrazine have a low degree of bioavailability in rats.

Pesticide residues in plants may be present in three possible forms: (i) freely extractable residues; (ii) extractable conjugates bound to natural components of plants; (iii) nonextractable or "bound" residues incorporated into the plant constituents. Many studies using radioisotopes as tracers within pesticide molecules have revealed that a considerable portion of pesticide residues, as much as 19-75% of the total ^{14}C in various crops, may become bound in plants (Khan, 1982; Huber and Otto, 1983). Bound residues in plants are difficult to identify and are not generally detected in routine residue analysis. Thus, for a long time the possible plant burden of total pesticide residues may have been underestimated. These bound and usually chemically unidentified residues may however be important. For example, they might be released on digestion of the contaminated food, become available for absorption in body tissues, and cause toxicity.

In earlier studies it has been demonstrated that bound pesticide residues in plants have low bioavailability to animals (Paulson et al., 1975; Sutherland, 1976; Dorough, 1976; Marshall and Dorough, 1977). These observations are based on the assumption that urinary and/or biliary excretion of the radioactivity from a radiolabeled pesticide or its metabolites indicates that the material is bioavailable. However, if the radioactivity is excreted quantitatively in the feces, it is not considered bioavailable. Paulson et al. (1975) fed bound residues in alfalfa plants treated with [^{14}C]phenyl propham to rats and found 93-95% radioactivity was excreted in the feces with only

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about 2% voided in the urine. Sutherland (1976) investigated the bioavailability of bound residues from [^{14}C]-phenylpropanil treated rice plants when fed to rats, dogs, and mice. It was observed that about 90% of the radioactivity was excreted in the feces, 10% in urine, and less than 0.05% in the bile. Marshall and Dorough (1977) investigated the bioavailability in rats of bound residues in bean plants treated with carbaryl and carbofuran. They observed that 98% and 85% of the radioactivity from the [^{14}C]carbaryl and [^{14}C]carbofuran treated plants were eliminated in the feces within 48 h, while urinary excretion amounted to only 1% and 11%, respectively. Based on this excretion pattern it was concluded that bound residues of carbamate insecticides in plants have a low degree of bioavailability.

None of the studies described above have reported on the chemical nature of the unidentified radioactivity fed to the animals in the form of bound residues. Furthermore, the identity of the radioactivity excreted in feces and urine remained unknown. Therefore, a need exists for additional studies which should include such information so that the question of bioavailability of bound residues could be assessed with greater certainty. In our experiments we attempted to study the potential bioavailability in rats of bound residues from corn plants treated with [^{14}C]atrazine. The chemical identity of radiolabeled bound residues in plant material before feeding to rats was determined. Furthermore, feces, urine, and body tissues were analyzed for residues. It was thought that the results of this study may help in evaluating the bioavailability of bound residues of known chemical identity and the possible toxicological significance of such residues.

EXPERIMENTAL SECTION

Chemicals. All solvents were of pesticide grade and used as received. Uniformly ^{14}C ring-labeled atrazine (specific activity 49.4 mCi/mmol, purity 98%), reference standards of atrazine, and metabolites were gifts from the Agricultural Division of Ciba Geigy Corporation, Greensboro, NC, and Basel, Switzerland.

Plant Material. Five vigorously growing corn (*Zea mays* L.) plants in a greenhouse, $2^1/_2$ months old, were removed from their pots, the roots were washed with cold tap water to remove soil, the plants potted in sand and irrigated with Hoagland's nutrient solution. The plants were grown in a controlled environment chamber with a relative humidity of 60%, and a 12-h photoperiod at 23 °C. After 8 days the plants were irrigated for 5 days with nutrient solution containing [^{14}C]atrazine (355 μCi/pot/plant) and unlabeled atrazine to give a final concentration of 5 ppm. The plants were then irrigated for 14 days with a nutrient solution containing 5 ppm of unlabeled atrazine and finally with a nutrient solution without herbicide for 3 days. At this stage the ears were full and well developed and the plants had matured. The control plants were grown under similar experimental conditions except that they received no herbicide treatment. At the end of the treatment, each plant was harvested and the aerial portion of plant was separated from the roots. The latter were washed with cold water, dried, and assayed for ^{14}C as described below.

Generation of Bound ^{14}C Residues in Plant. The aerial portion of the plant comprising of stem, leaves, ears, and tassel was blended at a high speed with dried chloroform (1:100 w/v) for 15 min. The mixture was filtered under suction and the sample residue washed with chloroform. The insoluble material was blended at high speed three times with methanol (1:100 w/v) for 15 min. Each time the mixture was filtered under suction, and the insoluble sample residue was finally blended with water (1:100 w/v). Further blending of the insoluble material with the solvent system did not result in any measurable extractable ^{14}C. The insoluble plant sample containing only bound ^{14}C residues was dried at 30 °C for 24 h and was divided into three parts. One part was combusted to $^{14}CO_2$ to determine the total bound ^{14}C. The other part was used to release bound ^{14}C by the high temperature distillation (HTD) technique described earlier (Khan and Hamilton, 1980). The distillates were subjected to various thin layer and column chromatographic procedures (Figure 1) and finally analyzed by gas chromatography (GC). In preliminary experiments it was observed that HTD of the solvent extracted nontreated corn plants to which atrazine and hydroxyatrazine was added (1.0 ppm) resulted in 79–92% recoveries of the compounds. Furthermore, analysis of the distillates (Figure 1) showed the presence of only the parent compounds and formation of any other breakdown products during distillation was not observed. The third portion of plant material containing bound ^{14}C residues was used for feeding to rats as described below.

Treatments of Rats and Collection of Samples. Sprague-Dawley rats weighing 161–190 g were housed, 1 animal/cage, in metabolism cages which separated urine and feces. They were conditioned for three days to a daily diet consisting of a mixture of rat feed (Purina Laboratory Chow) and nontreated extracted ground plant material (4:1 w/w). The latter in the mixture was then replaced for 2 days with either ground plant material containing bound ^{14}C residues (0.71×10^6 dpm/g) or [^{14}C]atrazine (0.52×10^6 dpm/g) fortified nontreated extracted sample. Eight rats were used in each treatment while 4 rats (control) continued to feed on the initial mixture. Each rat consumed about 12.5 g of ^{14}C material in the mixture of diet. The animals were then maintained on regular rat feed (Purina Laboratory Chow) for 3 days, the rats were sacrificed by decapitation, and selected organs were excised, weighed, and frozen for subsequent analysis. The urine and feces were collected daily after the treatment and radioassayed for ^{14}C. Portions of liver, kidney, and lungs were also combusted for determination of ^{14}C.

Analysis of Residues in Feces and Urine. (i) Feces. An aliquot of the feces of rats was extracted with chloroform and methanol until further extraction did not result in any extractable ^{14}C. The insoluble feces material containing only bound ^{14}C residues was dried in air and divided into two parts. One part was combusted to $^{14}CO_2$ for bound ^{14}C determination. The other part was used to release bound ^{14}C by the HTD technique (Khan and Hamilton, 1980), subjected to column cleanup, thin-layer chromatographic separation, and finally analyzed by GC as shown in Figure 2. **(ii) Urine.** The flow diagram in Figure 3 shows the procedure used to extract, isolate, and identify the ^{14}C residues from urine of rats. Preliminary experiments were carried out with urine from control rats, fortified with atrazine and hydroxyatrazine, in order to rationalize and validate the method. The recoveries from these experiments indicated that atrazine and hydroxyatrazine could be extracted in reasonably good yield (75 to 96%) by the procedure outlined in Figure 3.

Determination of Radioactivity. Liquids (e.g., urine, solvent extracts) were assayed in a Packard Model 3320 scintillation spectrometer and solids (plant material, feces, animal tissues) were assayed by combustion in a Packard sample oxidizer, Model 306.

Gas Chromatography (GC). The gas chromatograph was a Varian Model 6000 fitted with a thermionic-specific detector. The column was a 1.5m × 0.2cm i.d. glass tube

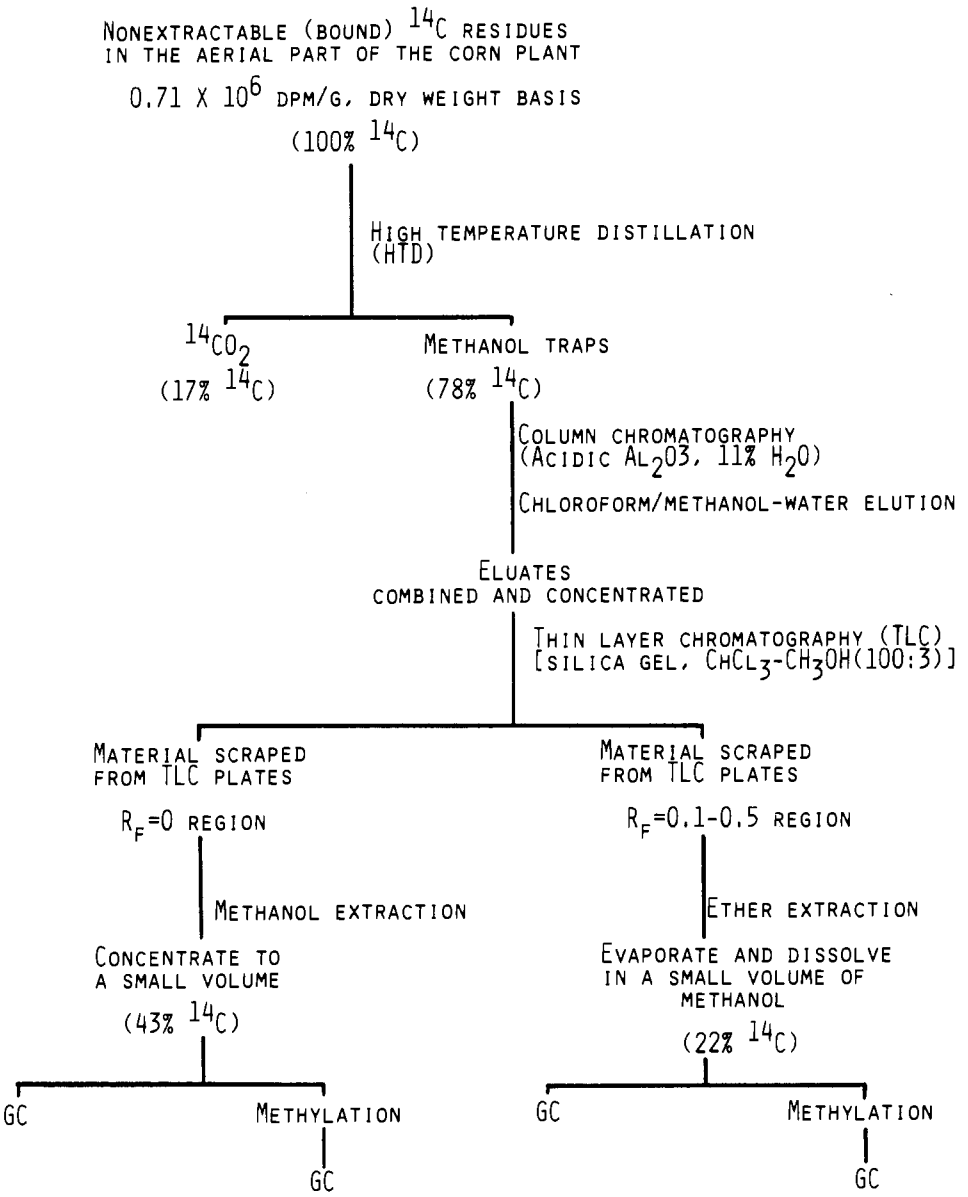


Figure 1. Schematic diagram for the analysis of nonextractable (bound) residues in corn plants treated with [^{14}C]atrazine.

packed with 3% Carbowax 3M coated on 100–120 mesh Supelcoport. The oven temperature was programmed at 1 °C/min from 180 °C to 215 °C and held at the latter temperature for 35 min. The detector and injector port temperatures were maintained at 290 °C and 215 °C, respectively; the helium carrier gas, hydrogen, and air flow rates were 30, 4, and 150 mL/min, respectively.

Confirmation. The identity of the compounds was confirmed by comparing the GC retention times with those of authentic samples, cochromatography, and finally by gas chromatography–mass spectrometry. A high-resolution mass spectrometer, Model VG 2AB-2F, connected to a Varian GC Model 3700 was used. The mass spectra were recorded at 70 eV.

RESULTS AND DISCUSSION

The corn plants contained 35% of the radioactivity applied in the nutrient solution. The roots and the aerial portion of the plants had 24% and 76%, respectively, of the total plant ^{14}C. Exhaustive extraction of the aerial portion of the plant with chloroform, methanol, and water removed about 84% of the total ^{14}C present whereas 16% of ^{14}C remained nonextractable (bound). The chloroform extracts comprised of less polar extractable residues contained a lower proportion of ^{14}C (20%) than the methanol–water extract containing more polar metabolites (64%). The chloroform or methanol–water extracts were not further analyzed in the present study.

Bound ^{14}C residues remaining in the solid material from the aerial portion of the plant after exhaustive solvent extraction were analyzed by the procedure shown in Figure 1. The major compounds identified were two mono-N-dealkylated analogues of hydroxyatrazine (VI, VII). Small quantities of hydroxyatrazine (II), atrazine (I), the two mono-N-dealkylated analogues of atrazine (III, IV), ammeline (V), and its hydroxy analogue (VIII) in the concentration range of 0.1–0.6 ppm were also detected in the HTD distillate of plants (Table I). Under the experimental conditions described about 65% of the bound ^{14}C residues in aerial portion of the corn plant were identified.

The radioactivity excreted in the urine and feces during the four day experiment is shown in Figure 4. The major route of excretion from rats fed plant material containing bound ^{14}C residues was in the feces. A reverse pattern of excretion of ^{14}C was observed when the corn material fortified with [^{14}C]atrazine was fed to rats. Thus, when the bound ^{14}C residues diet was given to rats, about 88% of the dose was eliminated in the feces at the end of 4 days,

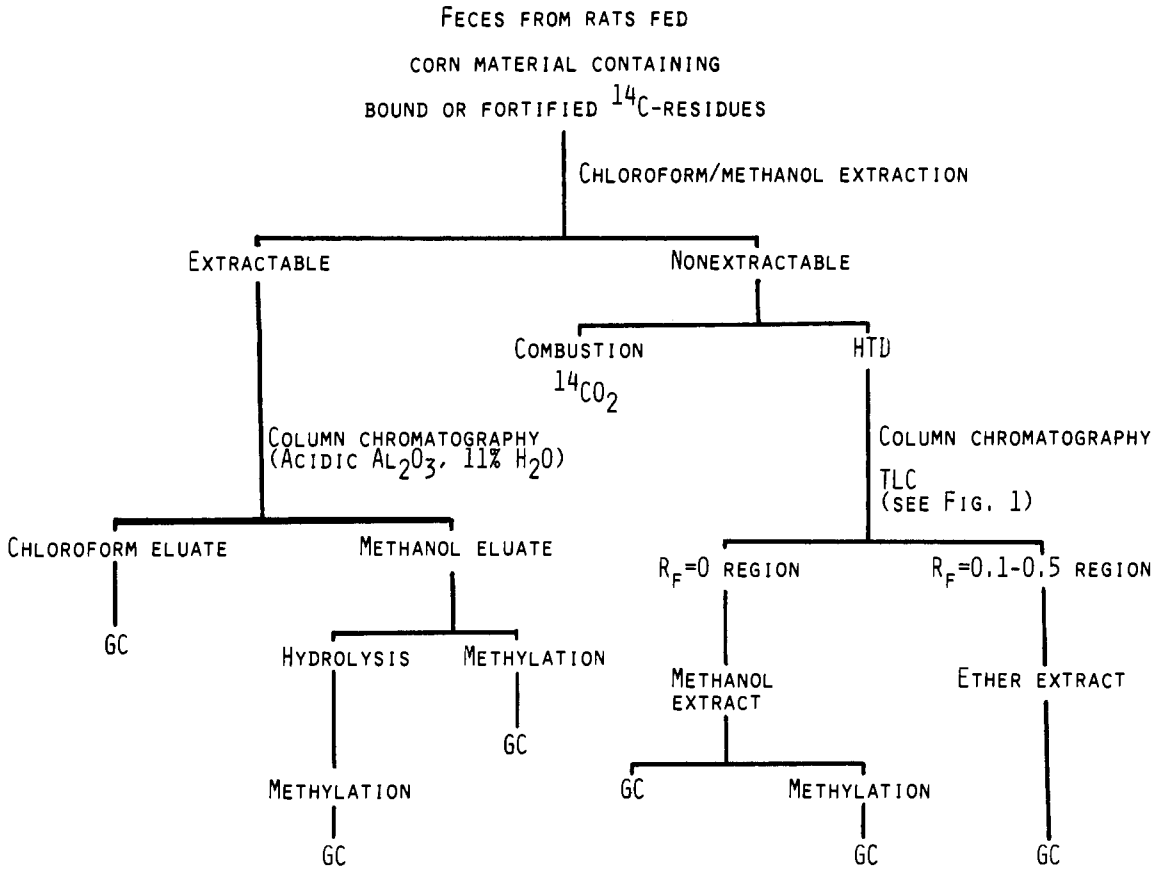
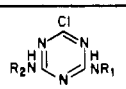
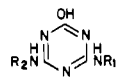
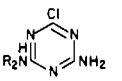
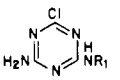
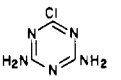
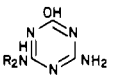
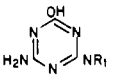
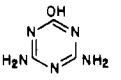


Figure 2. Schematic diagram for the analysis of feces from rats.

Table I. Bound Residues in Corn Material Fed to Rats and Analysis of Their Feces and Urine[a]

sample	 I	 II	 III	 IV	 V	 VI		 VIII
bound residue								
corn material	0.1	0.2	0.4	0.6	0.2	2.0	1.8	0.1
feces (A)	T	T	0.3	0.6	0.2	1.9	1.6	0.1
feces (B)	ND	ND	0.4	0.7	T	0.1	0.1	ND
extractable residues								
feces (A)	ND	T	ND	ND	ND	0.1	0.1	ND
feces (B)	ND	T	0.2	0.4	ND	0.6	0.5	ND
urine (A)	ND	ND	T	ND	ND	T	ND	T
urine (B)	ND	ND	2.4	1.6	0.5	T	T	T

[a] All values given in ppm. (A) Rats fed extracted corn material containing bound ^{14}C residues. (B) Rats fed extracted corn material containing fortified [^{14}C]atrazine. (T) Trace amounts <0.1 ppm. (ND) Not detected. R_1 = CH_3CH_2–; R_2 = $(CH_3)_2CH$–.

while the urinary excretion amounted to about 10%. However, rats given the [^{14}C]atrazine fortified diet eliminated about 60% and 32% radiocarbon in the urine and feces, respectively. These patterns of excretion from rats demonstrate the low degree of bioavailability of the bound ^{14}C residues in plants. However, freshly added [^{14}C]atrazine residues in plant material were far more readily metabolized and became bioavailable.

For the purpose of comparison, the radioactivity in tissues from rats given bound and fortified ^{14}C residues is shown in Table II. The bound residues had low bioavailability in rats as demonstrated by the low radiocarbon content in liver and kidney, and none in lungs. On the other hand, rats fed with [^{14}C]atrazine fortified diet showed relatively higher ^{14}C residues in these tissues (Table II).

Analysis of the extractable ^{14}C residues from the feces of rats fed bound ^{14}C residues according to the scheme shown in Figure 2 revealed the presence of small amounts of 2-OH analogues of deethylated and deisopropylated atrazine (VI, VII) and traces of hydroxyatrazine (II) (Table I). However, the extractable ^{14}C residues from feces of rats fed fortified diet contained relatively higher amounts of the two mono-N-dealkylated atrazine (III, IV) and their 2-OH analogues (VI, VII). Several other unknown small peaks in the gas chromatograms were also observed in the extracts of both type of feces (methanol eluates) but were not identified because of their low concentrations and the

Table II. Radioactivity in Tissues of Rats Fed Extracted Corn Plant Containing Bound or Fortified ^{14}C Residues

extracted corn containing ^{14}C	lungs		kidney		liver	
	weight,[a] g	^{14}C,[b] %	weight,[a] g	^{14}C,[b] %	weight,[a] g	^{14}C,[b] %
bound	0.9	ND	1.9	<0.1	9.4	<0.1
fortified	1.0	0.1	2.0	0.3	10.5	2.1

[a] Fresh weight in g (av of 8 rats). [b] Percent ^{14}C of the total ^{14}C consumed by each rat (≃12.5 g of ^{14}C material in the mixture of diet).

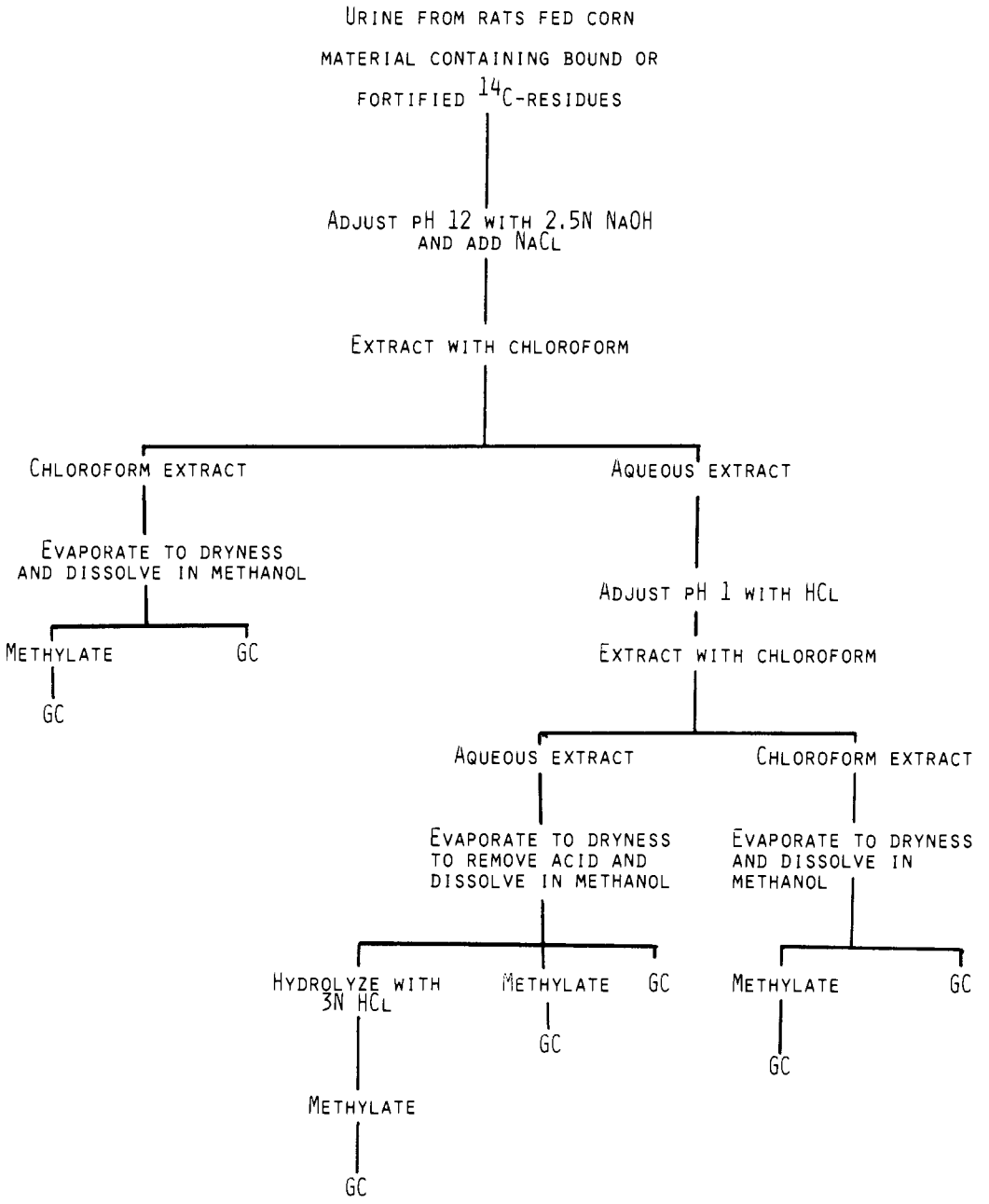


Figure 3. Schematic diagram for the analysis of urine from rats.

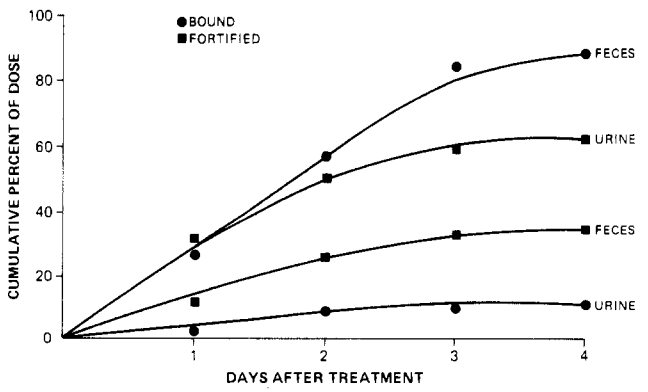


Figure 4. Elimination of radioactivity from rats fed corn material containing bound or fortified ^{14}C residues.

unavailability of reference standards.

GC analysis of the HTD distillates of extracted feces from rats fed bound ^{14}C residues indicated the presence of the two mono-N-dealkylated analogues of hydroxyatrazine (VI, VII) as the major compounds and small or trace amounts of other compounds shown in Table I. These compounds were also present in the extracted corn material containing bound ^{14}C residues. Furthermore, the amounts of these nonextractable compounds in feces, on a dry weight basis, were nearly equal to those observed in bound ^{14}C residue diet. It was observed that only a few of these products were extractable from the feces in small or trace amounts with the solvent system used (Table I) thereby indicating that most of the ^{14}C residues remained in the bound form. The urinary elimination of the ^{14}C residues did not exceed 10% of the dose in any of the rats fed bound ^{14}C residues. The excreted ^{14}C residues in urine analyzed by the scheme shown in Figure 3 revealed the presence of only trace amounts of deethylatrazine (III), its 2-OH analogue (VI), and 2-OH ammeline (VIII). It appears, therefore, that the extracted corn material containing bound ^{14}C residues did not undergo any appreciable biotransformation after it was consumed by rats as most of it was apparently not digested and was excreted in the feces. In contrast, GC analysis of the HTD distillates of the extracted feces from rats fed fortified diet indicated

the presence of the two non-N-dealkylated products of atrazine (III, IV) and their hydroxy analogues (VI, VII), whereas the presence of the parent herbicide (I) was not detected. It was also observed that about 60% of the radiocarbon was voided in the urine of rats fed [^{14}C]atrazine fortified corn material. Furthermore, relatively higher concentrations of the two mono-N-dealkylated atrazine metabolites, namely deethylatrazine (III) and deisopropylatrazine (IV), and ammeline (V) were found in the excreted urine (Table I). From these observations it is obvious that atrazine freshly added to the corn material before feeding was degraded effectively when consumed by rats. It should be realized that the procedures followed in this study were, of necessity, not typical of actual atrazine use conditions in the field. Therefore the values reported in this study should not be compared or extrapolated to field-grown corn due to the artificial conditions used.

It was observed that all of the 2-OH metabolites found in the aqueous extracts (Figure 3) of urine from both treatments were present in the form of conjugates as they were only released after hydrolysis. These polar metabolites may likely be present as glucuronide conjugates (Larson and Bakke, 1978) and must be considered highly bioavailable (Marshall and Dorough, 1977).

The results obtained in this study demonstrated a low degree of bioavailability in rats of the bound ^{14}C residues in corn plants treated with [^{14}C]atrazine. Unlike bound residues, freshly added [^{14}C]atrazine to plant material when fed to rats was readily bioavailable. This study also suggests that if we are to properly assess the bioavailability and/or toxicological significance of bound residues, information on their chemical identity must be obtained. In previous studies reported on the bioavailability of ^{14}C bound pesticide residues we do not know for certain whether these residues in the crop materials fed to animals, as well as the ^{14}C residues excreted, were present as the parent compound or in the form of their breakdown products.

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Registry No. Atrazine, 1912-24-9.

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Synthesis of (±)-7-Hydroxycostal and (±)-7-Hydroxycostol, Sweet Potato Phytoalexins

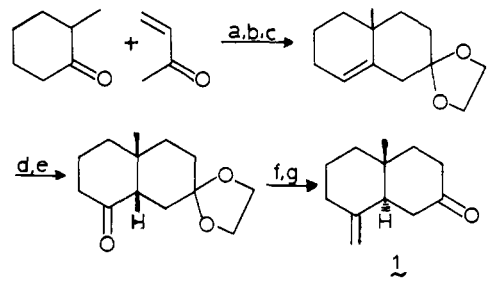
John Cuomo

The first synthesis of the sweet potato phytoalexins, 7-hydroxycostol and 7-hydroxycostal, by an eight- or nine-step sequence, respectively, is described. This synthesis confirms the structures proposed for the two defense compounds.

Recently, Schneider and Nakanishi (1983) isolated a new class of sweet potato phytoalexins containing the eudesmane skeleton. The defense compounds 7-hydroxycostal (**3**) and 7-hydroxycostol (**2**) accumulate in tissues infected with the black rot fungus Ceratocystis fimbriata and are reported to be potent fungal germination inhibitors. Nakanishi's structural analysis was based on evaluation of the NMR spectra of both the natural products and a semisynthetic derivative. The present work confirms this assignment through both total synthesis and X-ray crystallographic analysis and also provides a convenient, straightforward route to gram quantities of these novel compounds for complete biological evaluation.

The key intermediate **1** was synthesized by a modification of the literature procedure (Marshall and Fanta 1964; Marshall et al., 1966) as shown in Scheme I. When this method was used, the overall yield of **1** was 8% over seven steps from commercially available starting materials.

Scheme I[a]



[a] (a) EtOH/EtONa/−10 °C, 40%; (b) oxalic acid/water/reflux, 80–90%; (c) ethylene glycol/TsOH/toluene, 65–70%; (d) BH_3·THF; H_2O_2/NaOH, 95–100%; (e) pyridinium chlorochromate/CH_2Cl_2, 50–60%; (f) NaH/Me_2SO/methylenetriphenylphosphonium bromide, 70–75%; (g) 1 N HCl/silica gel/CH_2Cl_2, 90%.

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