photosynthetic processes take place. Plants kept in the dark contained less than $^1/_4$ of the amounts of radiocarbon taken up by plants exposed to light.

Registry No. Phorate, 298-02-2.

LITERATURE CITED

Bowman, B. T.; Sans, W. W. *J. Environ. Sci. Health, Part B* **1979**, *B14* (6), 625–634.
Derr, S. K.; Zabik, M. J. *Arch. Environ. Contam. Toxicol.* **1974**, *2* (2), 152–164.
Flashinski, S.; Lichtenstein, E. P. *Can. J. Microbiol.* **1974**, *20* (3), 399–411.
Hoagland, D. R.; Arnon, D. I. *Circ.—Calif. Agric. Exp. Stn.* **1950**, *No. 347*, 1–32.
Lichtenstein, E. P.; Fuhremann, T. W.; Schulz, K. R.; Liang, T. T. *J. Econ. Entomol.* **1973**, *66* (4), 863–866.
Lichtenstein, E. P.; Schulz, K. R.; Fuhremann, T. W. *J. Agric. Food Chem.* **1972**, *20* (4), 831–838.
Paris, D. F.; Lewis, D. L. *Bull. Environ. Contam. Toxicol.* **1976**, *15* (1), 24–32.
Schrader, G. "Die Entwicklung neuer insektizider Phophorsäure-Ester"; Verlag Chemie: Weinheim, West Germany, 1963; pp 393–405.
Walter-Echols, G.; Lichtenstein, E. P. *J. Econ. Entomol.* **1977**, *70* (4), 505–509.
Walter-Echols, G.; Lichtenstein, E. P. *J. Agric. Food Chem.* **1978a**, *26* (3), 599–604.
Walter-Echols, G.; Lichtenstein, E. P. *J. Environ. Sci. Health, Part B* **1978b**, *B13* (3), 149–168.

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In Vitro Release of Bound (Nonextractable) Atrazine Residues from Corn Plants by Chicken Liver Homogenate and Bovine Rumen Liquor

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The release of bound (nonextractable) residues from corn plants treated with [^{14}C]atrazine was investigated by in vitro incubation of the extracted plant tissue with chicken liver homogenate and bovine rumen liquor. Liver homogenate released bound ^{14}C residues from the plant tissues. However, no such release was observed with rumen liquor in the in vitro incubation system. The ^{14}C-bound residues in plant shoots or roots, mainly present as 2-chloro mono-N-dealkylated compounds, were released into the incubation mixture and subsequently metabolized to 2-hydroxy analogues.

Many studies have revealed that a considerable portion of pesticide residues may become bound (nonextractable) in plants (Fuhremann and Lichtenstein, 1978; Helling and Krivonak, 1978; Rouchaud et al., 1978, 1979; Fuhr and Mittelstaedt, 1980; Khan, 1980; Still et al., 1981). Oral administration of plant material containing ^{14}C-bound pesticide residues to animals has shown a rapid elimination of these residues via feces (Bakke et al., 1972; Paulson et al., 1975; Sutherland, 1976; Dorough, 1976; Marshall and Dorough, 1977). Thus, bound residues are considered to be of limited toxicological concern because of their limited bioavailability to animals.

In vitro studies have been undertaken to investigate the metabolism of pesticides by mammalian liver enzymes homogenates. Similarly, in vitro techniques in studies concerning metabolism of pesticides by rumen fluid have often been found useful. These methods applied to plant materials containing bound residues may provide useful information on the metabolic fate of these residues. Previously, we reported that the soluble fraction from chicken liver homogenates contained a heat-labile, glutathione-dependent enzyme that metabolized the herbicide atrazine in in vitro incubation (Foster et al., 1979). This paper reports the results of our investigation concerning the in vitro release of bound ^{14}C residues from corn plants by the chicken liver homogenates and rumen liquor from a lactating cow.

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EXPERIMENTAL SECTION

Plant Material. Ten corn plants (*Zea maise*), 19 days old, were exposed to 5 ppm of aqueous solution of ^{14}C-ring-labeled atrazine (0.16 Ci/jar). The plants were grown in Hoagland nutrient solution (300 mL/jar) and were maintained in a growth chamber for 8 days after herbicide treatment. Nutrient solution was added intermittently to the jar to replace loss due to transpiration over the growing period. At the end of the treatment period, each plant was harvested and the shoots and roots were separated. The latter was washed with cold water and the shoots and roots were stored at −20 °C until analyzed. Aliquots of the dried (24 h at 30 °C) plant tissues were combusted to $^{14}CO_2$ for determination of the total ^{14}C residues.

Chemicals. All solvents were of pesticide grade and used as received. Uniformly ^{14}C-ring-labeled atrazine, reference standards of atrazine, and metabolites were gifts from Ciba-Geigy, Ltd., Switzerland.

Determination of Radioactivity. Combustion of dried plant tissues was done in a Packard sample oxidizer, Model 306, to produce $^{14}CO_2$. Aliquots of various extracts (de-

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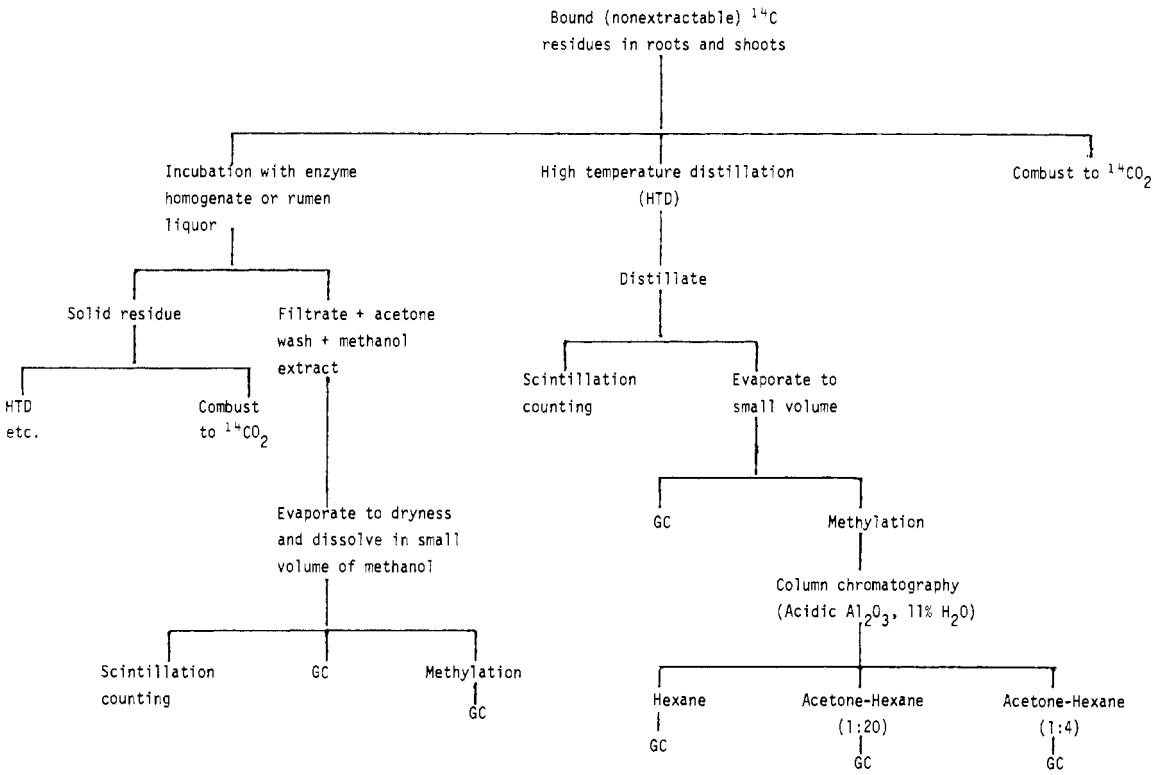

Figure 1. Schematic diagram for the analysis of bound ^{14}C residues in plant tissues and the extractable ^{14}C residues after incubation.

scribed later) and $^{14}CO_2$ released by combustion were analyzed by liquid scintillation counting (Khan and Hamilton, 1980).

Generation of Bound ^{14}C Residues in Plant Tissues. Both root and shoot samples were thawed at room temperature and blended at high speed with dried chloroform (1:100 w/v) for 5 min. The mixture was filtered under suction and the sample residue washed with chloroform. The insoluble material from root or shoot tissue was blended at high speed with methanol (1:100 w/v) for 5 min. The mixture was filtered under suction, and the insoluble sample residue was blended again with methanol (1:100 w/v) and finally with water (1:100 w/v). Further blending of the insoluble material with solvents did not result in any extractable ^{14}C. The insoluble shoot or root samples containing only bound ^{14}C residues were dried at 30 °C for 24 h and were divided into three parts. One part of shoot or root sample was combusted to $^{14}CO_2$ to determine the total ^{14}C. The other part was used to release bound ^{14}C by the high-temperature distillation (HTD) technique (Khan and Hamilton, 1980), subjected to column cleanup, and finally analyzed by gas chromatography as shown in Figure 1. The third portion was used for incubation studies as described below.

Preparation of Liver Homogenate and Rumen Liquor. A 20% (8 g of liver in 40 mL of buffer) homogenate of chicken liver was prepared in 0.134 M phosphate buffer, pH 7.4. The homogenate was centrifuged at 2500 rpm for 10 min at 4 °C. The liver homogenate thus prepared contained mitochondria, microsomes, and soluble fraction.

Rumen liquor obtained 3–4 h postfeeding from a fistulated cow fed corn silage was strained through four layers of cheesecloth. The filtrate was centrifuged at 1600 rpm for 20 min. The supernatant layer was decanted and used as inoculum media.

In Vitro Incubation. (i) Liver Homogenate. Ground plant material (100 mg) was suspended in 30 mL of freshly prepared liver homogenate in glass-stoppered Erlenmeyer flasks (50 mL) and incubated at 37.5 °C for 5 h. Control flask contained inactivated or no liver homogenate. In the latter case the volume was kept constant by adding an appropriate amount of buffer (30 mL). At the end of the experiment the enzymatic activity was destroyed by addition of 30 mL of acetone to the flasks. The mixture from each flask was filtered, the reaction flasks were rinsed with acetone (10 × 10 mL), and the rinse was again filtered and combined.

(ii) Rumen Liquor. Ground plant material (100 mg) was incubated with rumen liquor (50 mL) at 37.5 °C for 3 h. The control consisted of inactivated rumen liquor. At the end of the incubation period, the mixture was diluted to 100 mL with methanol and then centrifuged at 10 000 rpm. The supernatant was passed through filter paper.

Extraction and Determination of ^{14}C Residues. The analytical procedure used for the extraction, isolation and determination of ^{14}C residues after incubation is shown in Figure 1. The insoluble material from root or shoot tissue after incubation and filtration as described above was exhaustively extracted with methanol. The methanol extract was combined with the respective filtrate described above, evaporated to a small volume, and processed as shown in Figure 1. The residual extracted roots and shoots containing bound (nonextractable) residues were dried at 30 °C for 24 h and were combusted to $^{14}CO_2$ to determine the total ^{14}C.

Gas Chromatography (GC). The gas chromatograph was a Varian Model 6000 fitted with a thermionic-specific detector. The column was a 1.8 m × 0.2 cm i.d. glass tube packed with 3% Carbowax 20 M coated on 100–120-mesh Supelcoport. The operating conditions were as follows: column, detector, and injector port temperatures were 190, 300, and 220 °C, respectively; the nitrogen carrier gas, hydrogen and air flow rates were 20, 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

Table I. Distribution of ^{14}C Residues in Corn Plants

plant tissue	bound ^{14}C, %	extractable ^{14}C, % chloroform	methanol/water	total
root	15.3	5.9	27.8	33.7
shoot	22.9	8.9	19.2	28.1

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 51.9% of the ^{14}C applied in the hydroponic solution. Table I shows the distribution of ^{14}C residues in corn plants. The roots of corn plants contained 49.0% of the total plant ^{14}C whereas the remaining ^{14}C (51.0%) was present in the shoots. Amounts of total extractable ^{14}C residues were larger in roots but were smaller in shoots, accounting for 33.7 and 28.1%, respectively, of the total ^{14}C residues (Table I). The chloroform extracts (less polar residues) contained a lower proportion of ^{14}C than the methanol–water extracts (more polar residues).

Bound ^{14}C remaining in the solid material from roots and shoots after exhaustive solvent extraction amounted to 15.3 and 22.9%, respectively, of the total ^{14}C residues (Table I). GC analysis of the HTD distillates (Khan and Hamilton, 1980) indicated the presence of mono-N-dealkylated and traces of di-N-dealkylated compounds, namely, 2-chloro-4-amino-6-(isopropylamino)-*s*-trazine (deethylatrazine), 2-chloro-4-(ethylamino)-6-amino-*s*-trazine (deisopropylatrazine), and traces of 2-chloro-4,6-diamino-*s*-trazine (ammeline). Trace quantities of hydroxyatrazine and 2-hydroxy-4-amino-6-(isopropylamino)-*s*-trazine (deethylhydryoxyatrazine) were also detected in the HTD distillates of plant tissues. Under the experimental conditions described we were able to confirm the identity of about 80–85% of the bound ^{14}C residues in the plant tissue.

The solvents used and the extraction method employed in this study appeared to effectively remove all the extractable ^{14}C residues from plant tissues. However, incubation of plant tissues containing bound ^{14}C residues with buffer (pH 7.4) alone resulted in a further release of some ^{14}C residues (Table II). Incubation or extraction with aqueous buffers is not commonly employed in residue analysis methodology. In our study only the nonextractable ^{14}C residues in plant tissues after their exhaustive solvent extraction as described earlier were designated as bound residues. Thus, it appears that the ^{14}C residues released in buffer were less tightly bound than those remaining in the plant tissue matrix. Analysis of the material released by buffer from the root or shoot samples showed the presence of the 2-hydroxy analogue of deethylatrazine and traces of hydroxyatrazine.

The metabolism of atrazine in corn may involve glutathione conjugation (Shimabukuro et al., 1970) in addition to 2-hydroxylation and N-dealkylation (Shimabukuro, 1967). Some of the products thus formed may be incorporated into the natural constituents of plants such as lignin, starch, cellulose, and proteins to form bound residues (Khan, 1982). The bound residues resulting from the glutathione conjugation may likely be released by the action of liver homogenate. These types of conjugates are known to be cleaved by the liver enzymes (Akhtar, 1979; Hutson et al., 1976). In vitro incubation of the extracted plant tissues indicated that liver homogenate released

Table II. In Vitro Release of Bound ^{14}C Residues from Plant Tissue after Incubation with Liver Homogenate and Rumen Liquor[a]

incubation	^{14}C released, % of the total shoot	root
buffer	2.6	13.7
buffer plus liver homogenate	17.5	36.6
buffer plus deactivated liver homogenate	3.4	11.1
rumen liquor	4.4	11.8
deactivated rumen liquor	5.9	10.7

[a] Data are means from duplicate incubations.

bound ^{14}C residues (Table II). Thus, by the end of the 5-h incubation period with liver homogenate, 36.6 and 17.5% of the bound ^{14}C residues were released from roots and shoots, respectively. The corresponding release of ^{14}C residues with buffer alone or inactivated liver homogenate was considerably less (Table II). These observations are in agreement with an earlier study indicating the ineffectiveness of inactivated liver homogenate on the degradation of some insecticide (Akhtar, 1979). The amounts of bound ^{14}C in roots and shoots after incubation with liver homogenate were still higher, amounting to about 63.4 and 82.5%, respectively, of the originally bound ^{14}C residues in the plant tissue. The ineffectiveness of rumen liquor in releasing bound ^{14}C residues from the plant tissued was apparent in that only relatively small but similar amounts of extractable ^{14}C residues were present at the termination of incubation with both fresh and inactivated samples (Table II). Thus, the release of bound ^{14}C residues appears to be mainly due to the rumen liquor media (pH 6.5–6.9) rather than its action on cellulose containing ^{14}C residues. Cellulose is known to be hydrolyzed or digested by the microbial activity of rumen fluid (Johnson, 1966). Thus, it appears that no measurable ^{14}C residues were present in the cellulose fraction of the corn plant. In our studies incubation experiments were carried out for a relatively short duration as we were mainly concerned with the initially released materials rather than secondary products.

Analysis of the extractable ^{14}C residues from the incubation mixture of liver homogenate with the plant tissues revealed the presence of only 2-OH analogues of deethylated and deisopropylated atrazine. Furthermore, the root incubation mixture with liver also showed the presence of an additional di-N-dealkylated hydroxy compound, namely, 2-hydroxy-4,6-diamino-*s*-triazine. Extracts from the rumen liquor incubation samples contained traces of two dealkylated hydroxy metabolites. Several other unknown peaks were also observed in the extracts of the incubation mixtures of both liver homogenate and rumen liquor but were not identified because of their very low individual concentrations and the unavailability of their reference standards. In our preliminarly experiments low recoveries of hydroxy metabolites of atrazine were obtained due to poor efficiency of methylation (50–68%). Thus, no attempt was made to quantitate the residues identified in the extract of the incubation mixtures.

These studies indicate that the in vitro incubation of chicken liver homogenate with plant tissues containing bound ^{14}C residues released and metabolized some of these residues. However, no apparent release of ^{14}C residues was observed when the incubation involved rumen liquor. It appears that in the liver homogenate incubation system, the bound 2-chloro dealkylated metabolites in the plant tissues were released and subsequently metabolized to 2-hydroxy analogues. In our earlier studies it was shown that in the metabolism of atrazine by the soluble fraction

from chicken liver homogenates, the hydrolysis reaction predominates, resulting in the formation of 2-hydroxy analogues which do not undergo further degradation by dealkylation (Foster et al., 1980).

Only a few studies have been reported on the bioavailability of plant-bound residues to animals (Paulson et al., 1975; Sutherland, 1976; Dorough, 1976; Marshall and Dorough, 1977). It appears that bopund residues in plants are not available to the animals since, in nearly all the studies reported, elimination of bound ^{14}C residues has been found rapid and via the feces. Apparently, absorption of bound residues from the gastrointestinal tract will depend upon release of these residues from the plant matrix. The released residues will then accumulate in an effective concentration in solution at the site of absorption and subsequently result in permeation through the gastrointestinal barrier (Aguiar, 1975). This did not occur with the bound residues in plant material in the studies reported earlier (Paulson et al., 1975; Sutherland, 1976; Dorough, 1976; Marshall and Dorough, 1977). Our data showing no release of bound ^{14}C residues when incubated with rumen liquor substantiate the earlier findings.

Registry No. Atrazine, 1912-24-9; deethylatrazine, 6190-65-4; deisopropylatrazine, 1007-28-9; ammeline, 645-92-1; hydroxyatrazine, 2163-68-0; deethylhydroxyatrazine, 19988-24-0.

LITERATURE CITED

Aguiar, A. J. In "Pharmacology of Intestinal Absorption: Gastrointestinal Absorption of Drugs"; Forth, W.; Rummel, W., Eds.; Pergamon Press: New York, 1975; Vol. 1, p 335.

Akhtar, M. H. *J. Environ. Sci. Health, Part B* **1979**, *B14*, 53.

Bakke, J. E.; Shimabukuro, R. H.; Davison, K. L.; Lamoureux, G. L. *Chemosphere* **1972**, *1*, 21.

Dorough, H. W. In "Bound and Conjugated Pesticide Residues"; Kaufman, D. D.; Still, G. G.; Paulson, G. D.; Banadal, S. K., Eds.; American Chemical Society: Washington, DC, 1976; ACS Symp. Ser. No. 29, p 11.

Foster, T. S.; Khan, S. U.; Akhtar, M. H. *J. Agric. Food Chem.* **1979**, *27*, 300.

Foster, T. S.; Khan, S. U.; Akhtar, M. H. *J. Agric. Food Chem.* **1980**, *28*, 1083.

Fuhr, F.; Mittelstaedt, W. *J. Agric. Food Chem.* **1980**, *28*, 739.

Fuhremann, W.; Lichenstein, E. P. *J. Agric. Food Chem.* **1978**, *26*, 605.

Helling, C. S.; Krivonak, A. E. *J. Agric. Food Chem.* **1978**, *26*, 1163.

Hutson, D. H.; Holmes, D. S.; Crawford, M. J. *Chemosphere* **1976**, *5*, 79.

Johnson, R. R. *J. Anim. Sci.* **1966**, *25*, 855.

Khan, S. U. *J. Agric. Food Chem.* **1980**, *28*, 1096.

Khan, S. U. *Residue Rev.* **1982**, *84*, 1.

Khan, S. U.; Hamilton, H. A. *J. Agric. Food Chem.* **1980**, *28*, 126.

Marshall, T. C.; Dorough, H. W. *J. Agric. Food Chem.* **1977**, *25*, 1003.

Paulson, G. D.; Jacobsen, A. M.; Still, G. G. *Pestic. Biochem. Physiol.* **1975**, *5*, 523.

Rouchaud, J.; Dacallonne, J. R.; Meyer, J. A. *Pestic. Sci.* **1978**, *9*, 74.

Rouchaud, J.; Moons, C.; Meyer, J. A. *Pestic. Sci.* **1979**, *10*, 509.

Shimabukuro, R. H. *Plant Physiol.* **1967**, *42*, 1269.

Shimabukuro, R. H.; Swanson, H. R.; Walsh, W. C. *Plant Physiol.* **1970**, *46*, 103.

Still, G. G.; Balba, H. M.; Mansager, E. R. *J. Agric. Food Chem.* **1981**, *29*, 739.

Sutherland, M. L. In "Bound and Conjugated Pesticide Residues"; Kaufman, D. D.; Still, G. G.; Paulson, G. D.; Bandal, S. K., Eds.; American Chemical Society: Washington, DC, 1976; ACS Symp. Ser. No. 29, p 11.

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