Bound (Nonextractable) ^{14}C Residues in Soybean Treated with [^{14}C]Metribuzin†

Stéphane Dupont‡ and Shahamat U. Khan*,§

Land Resource Research Centre, Research Branch, Agriculture Canada, Ottawa, Ontario, Canada K1A 0C6

Bound residues of [^{14}C]metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one] in tolerant and susceptible soybean varieties (*Glycine max* L. Merr.) were studied. The plants were treated just before the flowering stage with radiolabeled herbicide and harvested on the 3rd, 10th, and 28th day after treatment. The roots, shoots, and beans of each plant were extracted with solvents and analyzed for bound ^{14}C residues. The tolerant variety contained relatively more bound ^{14}C fraction of the total ^{14}C in the plant than the susceptible. The bound residues were released from the extracted plant samples by high-temperature distillation and supercritical acetone extraction techniques. The distillates or the extracts were subjected to thin-layer chromatography, gas chromatography, and high-performance liquid chromatography for identification. Free [^{14}C]metribuzin and conjugated residues were shown to be present in the bound fraction of the roots and shoots of both varieties.

INTRODUCTION

Numerous pesticides are known to form bound (nonextractable) residues when applied to plants. Several workers have studied the fate of the herbicide metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one], a photosynthesis inhibitor, in soybean plants (Falb and Smith, 1984; Mangeot et al., 1979; Smith and Wilkinson, 1974). Soybean cultivars vary widely in their tolerance to metribuzin. Susceptible varieties appear to metabolize the herbicide very slowly, while tolerant varieties rapidly detoxify metribuzin by deamination to 6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one (DA) and conjugation with homoglutathione and glucose (Smith and Wilkinson, 1974; Mangeot et al., 1979; Fedtke and Schmidt, 1983; Falb and Smith, 1984; Frear et al., 1985). The presence of bound radioactivity in the metribuzin-treated soybean plants has been reported by various workers. Falb and Smith (1983) found relatively high levels of bound radioactivity in soybeans ranging from 18 to 47% of the total ^{14}C present in roots, stems, or leaves. Mangeot et al. (1979) reported relatively lower levels of bound radioactivity in resistant and susceptible varieties of soybean. However, these levels were higher in resistant plants than in susceptible plants, leading to the conclusion that the incorporation of metribuzin residues in the insoluble fraction may contribute to the tolerance of soybean plants.

In an attempt to identify the bound metribuzin residues in soybean, Frear et al. (1985) subjected extracted soybean plant material to acid hydrolysis. This procedure released about 40% of the bound ^{14}C in the form of 4-amino-6-(1,1-dimethylethyl)-1,2,4-triazine-3,5(2*H*,4*H*)-dione (DK). However, under the experimental conditions used, metribuzin would be hydrolyzed to DK, and it remained unclear whether or not metribuzin was present in the bound residue fraction.

High-temperature distillation and supercritical fluid extraction techniques have been used in our laboratory for releasing the bound (nonextractable) pesticide residues from biological samples. The present investigation reports on the applicability of these techniques in determining the distribution and nature of bound ^{14}C residues of metribuzin in susceptible and tolerant soybean varieties.

MATERIALS AND METHODS

Chemicals. Analytical grade metribuzin (purity 99.6%), [5-^{14}C]metribuzin (purity 98%, specific activity 21.9 mCi/mmol), and analytical grade DA, DK, and 6-(1,1-dimethylethyl)-1,2,4-triazine-3,5(2*H*,4*H*)-dione (DADK) with purities greater than 95% were gifts from Mobay Chemical Corp. All of the organic solvents used were of pesticide grade.

Plant Treatment. Two-week-old germinated seeds of susceptible (Maple Amber) and tolerant (Maple Arrow) soybean varieties were potted in coarse silica sand and grown in a controlled environment cabinet (relative humidity 60%, photoperiod 14 h, temperature 24 °C during light and 18 °C during dark). Initially half-strength Hoagland's nutrient solution (Hoagland and Arrow, 1950) was used, but on the fifth week after emergence, the concentration was increased to full strength. At the beginning of the seventh week [5-^{14}C]metribuzin in Hoagland's solution at predetermined tolerance threshold levels was applied to nine flowering plants of each variety: Maple Arrow (0.5 ppm, 4.12 μCi/L) for 3 days and Maple Amber (0.15 ppm, 3.92 μCi/L) for 4 days. Treatments were discontinued when the chlorophyll fluorescence measurements indicated the beginning of photosynthetic inhibition (Shaw et al., 1986).

After the last day of the radiolabeled herbicide treatment, the plants were subirrigated with Hoagland's nutrient solution and plants in triplicate (three plants) were harvested on the 3rd, 10th, and 28th day after the end of the treatment. In subsequent studies, each plant was processed separately, thereby providing three replicates for each sampling date. The beans, shoots, and roots of each plant were collected separately and frozen.

Control Maple Arrow and Maple Amber plants were cultured and harvested under the conditions identical to the treated plants with the exception that no [^{14}C]metribuzin was added to their nutrient solution.

Extraction and Determination of ^{14}C Residues. Each individual frozen plant samples was chopped separately into a blender container (Sorvall Omni-Mixer) and homogenized at a high speed for 5 min successively with 1:5 (fresh wt:vol) acetone, chloroform, and methanol. After each extraction, the sample was filtered under vacuum on Whatman No. 42 filter paper and washed with the same solvent. Further extraction with any one of these solvents did not result in any measurable increase of extractable ^{14}C. The extracts and washing were combined and concentrated on a rotary evaporator to a small volume and ra-

† Land Resource Research Centre Contribution No. 90-62.

‡ Graduate Student at the Department of Biology, University of Ottawa, Ottawa, ON, Canada.

§ Principal Research Scientist.

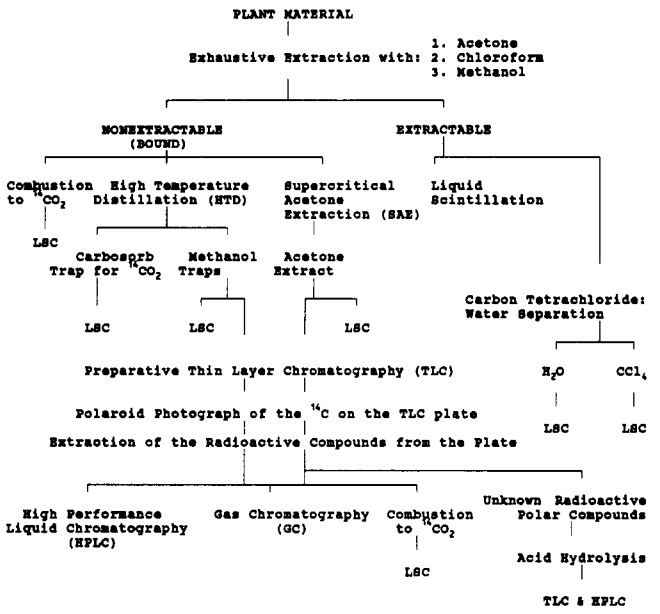

Figure 1. Flow diagram for the analysis of the bound residue of [^{14}C]metribuzin in soybeans.

dioassayed for ^{14}C. Dried subsamples of each fraction (insoluble plant material) were combusted in a Packard sample oxidizer, Model 306, to produce $^{14}CO_2$. The $^{14}CO_2$ was absorbed in Carbosorb (United Technologies Packard) and mixed with Permafluor V (U. T. Packard) for liquid scintillation counting. All scintillation counting was carried out with a Beckman Model LS 3801 counter (LSC). Figure 1 depicts analytical procedures.

Determination of Bound ^{14}C Residues. (*i*) *High-Temperature Distillation (HTD).* Each solvent-extracted sample containing bound ^{14}C residues was placed in a quartz tube and subjected to HTD as previously described (Khan and Hamilton, 1980). Weighed subsamples of approximately 1 g of roots or 2 g of shoots each were heated from room temperature to 600 °C in the quartz tube under a flow of helium (50 mL/min). The volatilized material was trapped in three methanol traps. An additional trap containing Carbosorb was used to absorb and quantitate any $^{14}CO_2$ produced in the process. The distillates were analyzed as shown in Figure 1 and described later.

(*ii*) *Supercritical Acetone Extraction (SAE).* An apparatus similar to that described by Capriel et al. (1986) was used. Weighed root (1 g) or shoot (2 g) subsamples of the solvent-extracted plants containing bound ^{14}C residues were placed directly in the sample holder (empty liquid chromatography column). Supercritical acetone (250 °C, 150 bar) was pumped through the apparatus at 1 mL/min for 3 h. The extracts were collected and analyzed as described below. Since typically high pressures are used in supercritical fluid extractions, caution should be exercised in handling flammable solvents under supercritical conditions.

The extracts obtained by procedures i and ii were concentrated and radioassayed.

Thin-Layer Chromatography (TLC). The TLC cleanup of extracts was carried out on preparative silica gel plates (Whatman PLK5F, 20 × 20 cm, 1000-μm thickness) with a solvent system of chloroform–dioxane (9:1). The concentrated distillate or extract was directly applied at the bottom of the central linear region of the TLC plate while vertical channels on the two sides were used for reference authentic compounds. The latter were located by observing the developed plate under UV light (λ = 254 nm) while the central portion was covered with aluminum foil to avoid any photodecomposition. Under these experimental conditions the R_f values of reference metribuzin, DA, DK, and DADK were 0.89, 0.66, 0.28, and 0.22, respectively. Polaroid photographs of the radioactive areas on the developed plates were taken with a Beta camera (Berthold, Model LB 292). The silica gel corresponding to the radioactive bands at R_f 0.88, 0.65, and 0.29 from the HTD distillates and the SAE extracts was scraped off the plate and extracted with acetone. The acetone extract was evaporated just to dryness, redissolved in methanol, and analyzed by liquid scintillation counting, gas chromatography, and high-performance liquid chromatography.

Gas Chromatography (GC). The gas chromatograph used was a Varian Model 3400 equipped with an SE-30 (coating thickness 0.25 μm) fused silica capillary column (0.25 mm i.d., 15 m long) and a thermoionic nitrogen-specific detector. The flow rate of the nitrogen carrier gas was 15 mL/min. The injector and detector temperatures were 190 and 285 °C, respectively. The column was conditioned for 24 h at 300 °C before the samples were injected. The column temperature was programmed from 140 to 190 °C at a rate of 3 °C/min. Under the GC conditions described, metribuzin, DA, DK, and DADK showed peaks at retention times of 7.0, 10.5, 5.0, and 3.7 min, respectively.

High-Performance Liquid Chromatography (HPLC). The high-performance liquid chromatograph used was a Varian Model 5500 equipped with a variable-wavelength UV detector (λ = 220 nm) and a radioactivity monitor and data station (Berthold LB 504 and LB 512). The column was a Whatman Partisil 10 ODS-2, particle size 10 μm (25 cm long, 4.6 mm i.d.), and the mobile phase was methanol–water (1:1) with a flow rate of 1 mL/min. The HPLC retention times of metribuzin, DA, DK, and DADK under these experimental conditions as determined by the UV detector were 18.1, 20.9, 8.1, and 10.2 min, respectively.

RESULTS AND DISCUSSION

Distribution of ^{14}C Residues in Soybean Plants. The distribution of bound ^{14}C residues in the soybean plants is shown in Table I. The roots of tolerant Maple Arrow plants contained a greater percentage of total plant ^{14}C as bound ^{14}C residues than those of the susceptible Maple Amber variety. However, there was no difference in the levels of bound ^{14}C residues in the shoots of the two varieties at any time after treatment. The main cause of the differential tolerance of soybean varieties to metribuzin has been shown to be the rate of metabolism of the herbicide (Smith and Wilkinson, 1974; Mangeot et al., 1979; Fedtke and Schmidt, 1983; Falb and Smith, 1984; Frear et al., 1985). However, greater amounts of bound residue formation in the roots of Maple Arrow variety may also contribute to its tolerance to metribuzin. The accumulation of metribuzin and/or metabolites in the roots may effectively prevent their translocation to the photosynthetic tissues of the leaves, where they would be expected to inhibit photosynthesis.

For both varieties the bound ^{14}C residues in beans represented less than 2% of the total ^{14}C in the plant. Furthermore, the shells (pericarps) contained more than 96% of the total ^{14}C present in the beans. Thus, the ^{14}C residues in seeds of both varieties represented less than 0.1% of the total ^{14}C in the plant. This suggests that soybean plants do not translocate a significant portion of metribuzin and/or metabolites residues to their seeds.

Recovery of [^{14}C]Metribuzin by HTD and SAE. The recovery of [^{14}C]metribuzin by HTD and SAE is shown in Table II. When pure metribuzin was processed by HTD or SAE, it was partially changed to DA. However, the presence of plant material induced a further partial degradation of metribuzin into DADK in addition to DA. Metribuzin was also thermally decomposed to CO_2 by HTD. The two metabolites, DA and DADK, were stable under the HTD experimental conditions with recoveries close to 100%, while the third metabolite, DK, was recovered only up to 41%. However, in the presence of plant material complete decomposition of these metabolites occurred by HTD. No measurable amounts of intact DA, DK, or DADK were recovered by SAE when processed alone or in the presence of plant material. The reason for the partial or complete breakdown of metribuzin and/or its metabolites during HTD or SAE in the presence of plant matrix will remain a matter of conjecture until more

Table I. Distribution of Bound ^{14}C Residues in Roots, Shoots, and Beans of [^{14}C]Metribuzin-Treated Soybean Plants

	bound ^{14}C as % of total ^{14}C[a] at days after treatment					
	3		10		28	
plant part	Maple Arrow (R)[b]	Maple Amber (S)[c]	Maple Arrow (R)	Maple Amber (S)	Maple Arrow (R)	Maple Amber (S)
shoots	29.0 ± 6.3	24.4 ± 4.5	34.7 ± 8.4	34.7 ± 8.2	39.7 ± 4.1	42.6 ± 0.9
roots	13.8 ± 5.3	7.4 ± 1.2	21.0 ± 5.1	9.3 ± 3.9	16.1 ± 1.3	7.4 ± 1.7
beans	included with shoots		1.0 ± 0.6	1.3 ± 1.2	1.5 ± 0.4	1.8 ± 0.1

[a] Average of three replicates ±95% confidence interval. [b] R, tolerant variety. [c] S, susceptible variety.

Table II. Recovery of [^{14}C]Metribuzin by High-Temperature Distillation (HTD) and Supercritical Acetone Extraction (SAE)

sample	compound recovered	% of the applied HTD	% of the applied SAE
[^{14}C]metribuzin	[^{14}C]metribuzin	78	78
	$^{14}CO_2$	2	ND[a]
	[^{14}C]DA	9	12
control plant material fortified with [^{14}C]metribuzin	[^{14}C]metribuzin	30	52
	$^{14}CO_2$	11	ND
	[^{14}C]DA	13	22
	[^{14}C]DADK	14	19

[a] ND, not detected

Table III. $^{14}CO_2$ and Methanol-Soluble ^{14}C Residues Released by High-Temperature Distillation of the Soybean Plant Samples

harvest time, days	% of the total ^{14}C recovered by HTD: Maple Arrow (R)[a] $^{14}CO_2$	Maple Arrow (R)[a] methanol-soluble ^{14}C	Maple Amber (S)[b] $^{14}CO_2$	Maple Amber (S)[b] methanol-soluble ^{14}C
3	42.8	57.2	18.9	51.1
10	51.1	48.9	27.8	72.2
28	58.1	41.9	31.9	68.1

[a] R, tolerant variety. [b] S, susceptible variety.

information is available. However, judging from these results, it is obvious that the recovery data obtained in our experiments cannot be used to extrapolate the actual amounts of bound residues present in the treated plant samples.

Release of Bound ^{14}C Residues and Their Analyses. All soybean samples, except the beans which did not contain sufficient bound radioactivity to allow their analysis, were processed by HTD. For the 22 samples analyzed the average recovery of ^{14}C in trapping solvents at ±95% confidence interval was 84.9 ± 2.0% of the total ^{14}C in the plant tissue. The proportions of $^{14}CO_2$ and methanol-soluble ^{14}C compounds collected are shown in Table III. These amounts were not significantly different in the two varieties between the root and shoot of each plant sample, as determined by Student's t-test. Thus, the data in Table III are the average values for each plant. The $^{14}CO_2$ production during HTD increased with the time of harvest for both varieties and was greater in Maple Arrow than in Maple Amber samples. It is likely that ^{14}C residues bound in plant material for a longer time may be more deeply embedded or entrapped in the cell wall matrix. Increase in temperature during HTD will result in decomposition or degradation of plant macromolecules. This in turn would likely release the deeply embedded or entrapped ^{14}C residues. At higher temperatures the released ^{14}C residues would more likely decompose to $^{14}CO_2$. It was observed that during the early period of growth after treatment Maple Arrow appeared to have a greater amount of bound residue formation than Maple Amber (Table I). This would obviously result in the formation of more "older" bound residues during the entire growth period in the tolerant variety than in the susceptible variety.

Table IV. Comparison of Radioactivity Levels among Fractions Obtained at Different R_f Values from the TLC Analyses of High-Temperature Distillates (HTD) and Supercritical Acetone Extracts (SAE)

R_f value	% of total ^{14}C in the three fractions: HTD shoots	HTD roots	SAE shoots	SAE roots	compounds identified by HPLC with monitor
0.88	12.6	7.0	22.8	16.5	metribuzin
0.65	43.2	25.5	38.8	25.2	DA + unknown
0.29	44.1	67.5	38.4	58.3	DADK

The HTD distillates of plant materials required cleanup before analysis by HPLC. The concentrated distillate in methanol was subjected to TLC separation as described earlier. A photograph of the TLC plate by Berthold Beta camera showed radioactive regions at R_f 0.88, 0.65, and 0.29. The radioactive regions on TLC plates were scraped off the plate, extracted with methanol, and radioassayed for ^{14}C (Table IV). HPLC analysis of the extracts showed peaks at 18.1 (R_f 0.88), 20.9 (R_f 0.65), and 10.2 min (R_f 0.29), which were identified by comparison of the retention time of authentic reference standards as metribuzin, DA, and DADK, respectively. The identity of these compounds was further confirmed by GC analysis of the HTD extracts and by cochromatography with the authentic standards. It was observed that the relative proportions of [^{14}C]-metribuzin (R_f 0.88) and [^{14}C]DA (R_f 0.65) were greater in shoots than in roots for both varieties. However, more [^{14}C]DADK residues (R_f 0.29) were present in roots than in shoots.

The SAE extract contained an average of 69.2% of the total bound ^{14}C in the sample, while the remaining ^{14}C remained unextracted from the plant material. Thin-layer chromatography of the concentrated SAE acetone extracts showed three radioactive bands at R_f values similar to that observed for HTD distillates (Table IV). However, an additional radioactive band at R_f 0.0–0.2 was also observed on TLC. The HPLC and GC analyses of the extracts of the scraped materials from the three bands (R_f 0.88, 0.65, and 0.29) confirmed the identity of metribuzin, DA, and DADK. In addition, the presence of some unknown ^{14}C products was also observed in the extract of scraped material at R_f 0.65 (Table IV).

The extracts from the radioactive region (R_f 0.0–0.2) eluted on HPLC as a broad and irregular peak at R_t of approximately 1 min. This peak represented 72.1 and 49.6% of the ^{14}C extracted from Maple Arrow and Maple Amber samples, respectively. It was of interest to note that the greater release of the unknown polar compounds ($R_t \simeq 1$ min) by SAE corresponded to the increased production of $^{14}CO_2$ during HTD of Maple Arrow samples. The unknown polar products probably contained unreleased ^{14}C residues in the form of conjugates. The material was hydrolyzed with an aqueous solution of 1 N HCl as described before. The solution was extracted with chloroform. The aqueous phase retained 58% of the initial

radioactivity, while the organic phase contained 42% of ^{14}C. TLC (chloroform–dioxane = 9:1) of the latter resulted in two radioactive bands at R_f 0.0–0.2 (26% ^{14}C) and 0.3 (11% ^{14}C). The identity of ^{14}C residue in the methanol extracts of these bands could not be established by HPLC due to low radioactivity levels. However, TLC analysis indicated that the compound at R_f 0.3 was likely to be DADK. These results show that the ^{14}C label of at least some of these unknown polar compounds remained a part of a distinct herbicide residue moiety conjugated to an unidentified endocon moiety. It is not known whether that herbicide residue moiety (exocon) was metribuzin or the metabolite DADK since metribuzin was shown to decompose to DADK during SAE. A large fraction of unknown polar compounds did not release ^{14}C label oxocon by hydrolysis. It is likely that the release of ^{14}C label oxocon was prevented by the rearrangement and/or modification of the conjugates during SAE. It has been suggested that often a considerable portion of bound ^{14}C residues in plants may be released by supercritical methanol extraction as unknown polar metabolites which could be included in macromolecular type structures (Capriel et al., 1986). Thus, it appears that supercritical fluid extraction of plant-bound residue fraction may release some pesticide residues in the form of exocons conjugated with cell wall polymers in addition to the free parent compound and/or metabolites.

In a recent paper Frear et al. (1985) reported on the acid hydrolysis of the bound residue fraction of [^{14}C]metribuzin-treated excised soybean leaves. This procedure released about 40% of the bound residues as the metabolite DK. Since DK is also a product of acid hydrolysis of metribuzin, it remained unclear whether the bound residue was the parent compound or its diketo metabolite.

Conclusion. The data presented in this study show that metribuzin was present in the bound residues fraction of both resistant and susceptible varieties of soybean. In addition to metribuzin, free DA and DADK metabolites were also released by HTD and SAE of the plant material containing bound residues. Furthermore, a large fraction of the ^{14}C residues was recovered as $^{14}CO_2$ after HTD. The results suggest that both metabolites and $^{14}CO_2$ originated, at least in part, from the transformation of bound metribuzin during the HTD or SAE process. A comparison of the bound ^{14}C residues data obtained by these two techniques shows that larger amounts of metribuzin and DA were recovered from shoots than from roots. However, more DADK was present in roots. The results also suggest that the higher proportions of bound residue formation in the roots of Maple Arrow than in Maple Amber may contribute to the differential tolerance of the two varieties.

ACKNOWLEDGMENT

S.D. gratefully acknowledges financial assistance from the National Science and Engineering Research Council of Canada during the course of the research. This study was a part of a coordinated program of research under the sponsorship of the International Atomic Energy Agency, Vienna, Austria.

LITERATURE CITED

Capriel, P. A.; Haisch, A.; Khan, S. U. Supercritical methanol, an efficacious technique for the extraction of bound pesticides residues from soil and plant samples. *J. Agric. Food Chem.* **1986**, *34*, 70–73.

Falb, L. N.; Smith, A. E., Jr. Metribuzin metabolism in soybeans. Characterization of the intraspecific differential tolerance. *J. Agric. Food Chem.* **1984**, *32*, 1425–1428.

Fedtke, C.; Schmidt, R. R. Behavior of metribuzin in tolerant and susceptible soybean varieties. In *Pesticides Chemistry: Human Welfare and the Environment*; Miyamoto, J., Kearney, P. C., Eds.; Pergamon Press: New York, 1983; pp 177–182.

Frear, D. S.; Swanson, H. R.; Mansager, E. R. Alternate pathways of metribuzin in soybean. Formation of N-glucoside and homoglutathion conjugates. *Pestic. Biochem. Physiol.* **1985**, *23*, 56–65.

Hoagland, D. R.; Arnon, D. I. The water culture method for growing plants without soil. *Circ.—Calif. Agric. Exp. Stn.* **1950**, *No. 547*.

Khan, S. U.; Hamilton, H. A. Extractable and bound (nonextractable) residues of prometryn and its metabolites in an organic soil. *J. Agric. Food Chem.* **1980**, *28*, 126–132.

Mangeot, B. L.; Slife, F. E.; Rieck, C. E. Differential metabolism of metribuzin by two soybean (Glycine max) cultivars. *Weed Sci.* **1979**, *27*, 267–269.

Shaw, D. R.; Peeper, T. F.; Nofziger, D. L. Evaluation of chlorophyll fluorescence parameters for an intact plant bioassay. *Crop Sci.* **1986**, *26*, 756–760.

Smith, A. E., Jr.; Wilkinson, R. E. Different absorption, translocation and metabolism of metribuzin in soybean cultivars. *Physiol. Planta* **1974**, *32*, 252–257.

Received for review August 19, 1991. Revised manuscript received November 13, 1991. Accepted March 3, 1992.

Registry No. DA, 35045-02-4; DK, 56507-37-0; DADK, 52236-30-3; metribuzin, 21087-64-9.