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Mode of Dinitroaniline Herbicide Action

II. CHARACTERIZATION OF [^{14}C]ORYZALIN UPTAKE AND BINDING[1]

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ABSTRACT

The intracellular binding of dinitroaniline herbicides was studied in order to analyze the mechanism of their colchicine-like action. When corn root apices (5 millimeters) are incubated in [^{14}C]oryzalin (a dinitroaniline herbicide), the ^{14}C is taken up rapidly, reaching a plateau in about 4 hours, which corresponds to the minimum incubation time in oryzalin required to get maximum inhibition of elongation. At 4 hours, the [^{14}C]oryzalin concentration inside the roots is 35 times higher than that in the incubation medium. Since this accumulation of [^{14}C]oryzalin is not affected by 1 millimolar sodium azide and there is no metabolism of [^{14}C]oryzalin under these conditions, the [^{14}C]oryzalin must be accumulated (bound) in corn root apices by a process not requiring metabolic energy.

Molecular sieve chromatography (Sephadex G-200) does not show any binding of [^{14}C]oryzalin to any protein with molecular weight similar to the microtubule-subunit protein in rat brain or corn root extracts. However, a massive binding of [^{14}C]oryzalin occurs in the insoluble fraction of corn root extracts. This binding is not localized in any particle size range, is not affected by sonication, is of high capacity, and is a loose (low affinity) association with the binding sites. These binding sites could be solubilized with membrane detergents, which suggests that oryzalin may bind to cellular membranes. Since boiling the homogenate does not affect [^{14}C]oryzalin binding, oryzalin more likely binds to a lipid rather than a protein component of cellular membranes.

The herbicidal activity of DNHs[3] results from their effect on the pre-emergence stage of plant growth (6). Although shoots of monocots and hypocotyls of dicots are suggested sites of DNH uptake (1, 6), their uptake by roots and effect on root development seems to be crucial to their herbicidal action. When applied to roots, DNHs, such as benefin (5), oryzalin (8), and trifluralin (3), are accumulated in roots with very little transport to the shoots (6, 8). However, very little is known about their intracellular localization within roots or about their mode of action.

Because of several similarities between the effects of colchicine and DNHs (*viz.* change in the polarity of cell enlargement, disruption of mitosis, etc.), it is generally believed that DNHs act like colchicine (see ref. 8 for discussion), *i.e.* by interacting with microtubule subunit protein. Hess and Bayer (4) reported that the DNH trifluralin bound to a tubulin-like protein from *Chlamydomonas* flagella. However, to date, all attempts to demonstrate such an interaction in higher plants have been unsuccessful (2, 9), and it is still uncertain whether DNHs act by interacting with microtubule subunit protein or by some other mechanism. Understanding the mode of DNH action is of interest not only because of their herbicidal action but also because they could be useful probes of microtubule function and control of the polarity of cell enlargement.

Here, the uptake of oryzalin and its binding in the soluble and insoluble fractions of corn root extracts are examined with the aim of analyzing the mode of DNH action.

MATERIALS AND METHODS

Plant Material. Five-mm corn (*Zea mays* L., yellow field corn, Michigan 200 hybrid) root apices, excised from 48-h-old corn seedlings (8), were used in all experiments.

[^{14}C]Oryzalin Uptake. Fifteen or 30 root apices were incubated in 10 ml incubation buffer [5 mM KH_2PO_4, 2% w/v sucrose (pH 5.5)] for 3 h (in 50-ml Erlenmeyer flasks at 25 C in a rotary water bath shaker in darkness) to allow the root apices to adjust to the new conditions. The [^{14}C]oryzalin (9.44 μCi/mg, stock solution in ethanol) was added. Uptake was generally measured by periodical sampling (0.2-ml samples) of the ambient solution as described by Upadhyaya and Noodén (8), but sometimes root extracts were made as direct checks of uptake. To study the effect of the respiratory inhibitor sodium azide on oryzalin uptake, root apices were first incubated in 1 mM sodium azide for 4 h, then [^{14}C]-oryzalin was added and uptake was studied as above. Oryzalin (3,5-dinitro-N^4, N^4-dipropylsulfanilamide) and [^{14}C]oryzalin were donated by Lilly Research Laboratory, Greenfield, Ind, and A-820 (*N-sec*-butyl-4-*tert*-butyl-2,6-dinitroaniline) was donated by Amchem Products Inc., Ambler, PA.

Metabolism of [^{14}C]Oryzalin. Root apices (300) were incubated in 50 ml incubation buffer containing 1 μM [^{14}C]oryzalin. After 12 h incubation, roots were ground in a Potter-Elvehjem homogenizer and extracted with 10 ml acetone for about 12 h in darkness in a rotary shaker bath at 25 C. The particulate material was removed by centrifugation, washed twice with 5 ml acetone, and all extracts were pooled. The acetone extract was concentrated, co-spotted with unlabeled oryzalin on a Gelman ITLC-SA (Gelman Instrument Co., Ann Arbor, MI) plate, and chromatographed using ethyl acetate-benzene (5:50, v/v) or *n*-heptane-isopropanol-methanol (7:1:2, v/v) as solvents.

Binding in Soluble Fraction. Three hundred 5-mm root apices (2.2 g) were incubated in 50 ml of 10 μM [^{14}C]oryzalin at 25 C for 4 h. Then root apices were homogenized at 4 C in 3.25 ml homogenization buffer [0.1 M NaH_2PO_4, 0.4 M sucrose, 1 mM each of $MgCl_2$ and cysteine, 200 μM phenylmethylsulfonyl fluoride (pH 6.8)] containing 10 μM [^{14}C]oryzalin. The cold homogenate was filtered through two layers of cheesecloth and centrifuged at 200,000*g* (SW 50.1 rotor) for 1 h at 2 C. One ml supernatant was

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[3] Abbreviation: DNH, dinitroaniline herbicide; EGTA, ethylene glycol *bis*(β-amino ethyl ether)-*N,N'*-tetraacetic acid.

loaded onto a Sephadex G-200 column (1.2 × 18.5 cm) and developed at 5 to 10 ml/min at 4 C. A_{230} and radioactivity were measured for each fraction.

One fresh rat brain [a rich source of microtubule subunit protein tubulin (12)] was ground in homogenization buffer (1 g brain: 1.25 ml buffer) as above. The homogenate was filtered through cheesecloth and centrifuged (200,000*g*) for 1 h. One-half ml of the postribosomal fraction (200,000*g* supernatant) was incubated with 10 μl of 0.2 mM [^{14}C]oryzalin at 37 C for 1 h and fractionated on the Sephadex G-200 column described above. [^{3}H]Colchicine (New England Nuclear, 11.18 Ci/mmol) binding in this postribosomal fraction was also studied by the procedure described above.

Binding in Insoluble Fraction. Two ml assay homogenate (details are given with the relevant tables and figures) were centrifuged at 200,000*g* for 1 h (2 C) in 2-ml cellulose nitrate tubes. The supernatant was decanted, and the bottom of the tube with the pellet was cut out. The excess liquid was absorbed with filter paper, and the radioactivity in the pellet was determined after resuspension in 1 ml of H_2O.

Measurement of Radioactivity. By liquid scintillation counting as described previously (8).

RESULTS

Uptake and Release of [^{14}C]Oryzalin. [^{14}C]Oryzalin was taken up rapidly by the root apices and reached a plateau in about 4 h (Fig. 1); there was no further increase in ^{14}C levels for up to 12 h incubation. Since the fresh weight of root segments steadily increased (Fig. 1) during this 12-h period, the [^{14}C]oryzalin uptake/g fresh weight declined slightly after 4 h incubation. The accumulation ratio (cpm per g fresh weight/cpm per ml incubation medium) also increased for 4 to 5 h and then stayed at about 35. Similarly, when intact corn seedling roots were immersed in 1 μM [^{14}C] oryzalin solution, the [^{14}C]oryzalin accumulated inside roots with almost no transport to the shoots in 48 h (8).

Uptake and accumulation of [^{14}C]oryzalin by the root tip segments was not inhibited by 1 mM sodium azide treatment; in fact, sodium azide caused a small increase in [^{14}C]oryzalin uptake. TLC of the acetone extract (which contained 99% of the [^{14}C]oryzalin taken up) using two different solvents showed that only one peak of radioactivity was present and that it moved at the R_F of nonradioactive oryzalin.

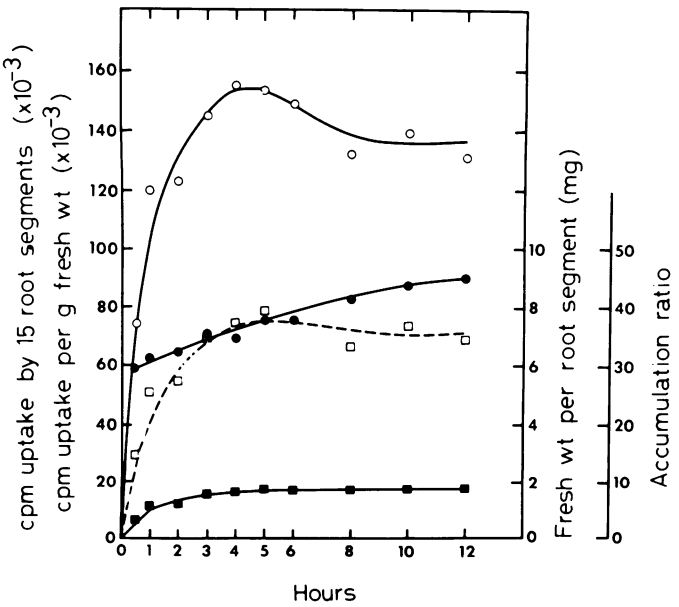

FIG. 1. Time course of [^{14}C]oryzalin uptake by apical 5-mm corn root segments. (■——■), cpm taken up by 15 root segments; (●——●), mg fresh weight/root segment; (○——○), cpm uptake/g fresh weight; (□- - -□), accumulation ratio (cpm per g fresh weight/cpm per ml incubation medium).

When root segments incubated in 1 μM [^{14}C]oryzalin for 4 h were transferred to 50 ml incubation buffer with or without 10 μM nonradioactive oryzalin in a rotary water bath shaker (25 C), the ^{14}C was released rapidly (Fig. 2); the cpm/g fresh weight decreased by about 64% at the end of 4 h washing. This release of ^{14}C was not affected by the presence of nonradioactive oryzalin in the washing buffer.

Dose Effects on Uptake. When the root segments were incubated in 0.25 to 6 μM [^{14}C]oryzalin, there was a rapid initial uptake with accumulation reaching a plateau by 4 h at all concentrations. This concentration range was determined by proximity to the solubility limit (about 6 μM) and the detection limit established here (0.25 μM). Both the initial rate of uptake (cpm/g fresh weight in 30 min) and accumulation (cpm/g fresh weight at 4 h) increased with increasing concentrations (Fig. 3a). The [^{14}C]oryzalin concentration for half-maximum accumulation at 4 h, calculated from double-reciprocal plot, was 21 μM (Fig. 3b). The best-fitting straight lines in the double-reciprocal plots were determined by linear regression analysis. Although the double-reciprocal plots suggest the presence of only one kind of binding in the insoluble fraction, the possible existence of a small capacity, higher affinity binding is not yet excluded. Investigation of such binding was not possible due to low specific radioactivity of the [^{14}C]oryzalin available (see ref. 7 for discussion).

Binding in Soluble Fraction. When the 100,000*g* supernatant of corn root extract was incubated with 10 μM [^{14}C]oryzalin at 37 C for 1 h and the incubation mixture was fractionated on a Sephadex G-200 column, no [^{14}C]oryzalin binding was observed. However, in other experiments, Sephadex G-200 elution profile of the postribosomal (200,000*g*) supernatant obtained from roots that

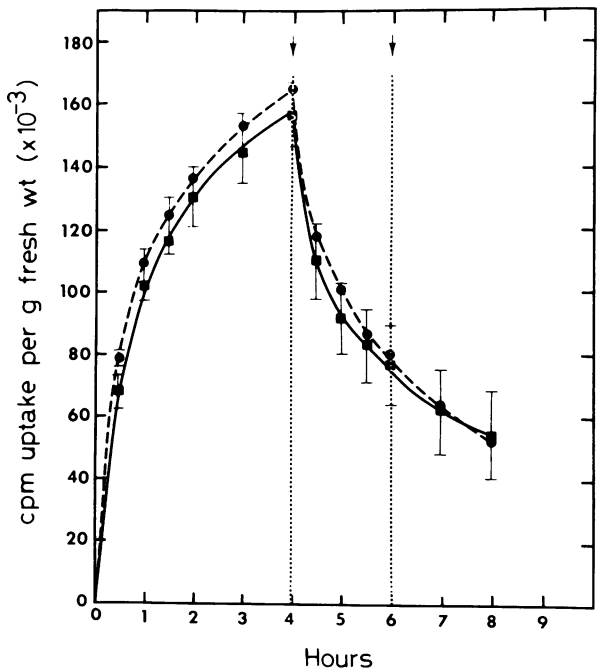

FIG. 2. Uptake and release of [^{14}C]oryzalin by 5-mm corn root segments after transfer to incubation buffer with (●- - -●) or without (■——■) nonradioactive oryzalin. For the first 4 h in both treatments, the root segments (15) were incubated in 1 μM [^{14}C]oryzalin without any nonradioactive oryzalin. Arrows indicate the times of transfer to buffer with or without 10 μM nonradioactive oryzalin. Each point is the mean of three replicates.

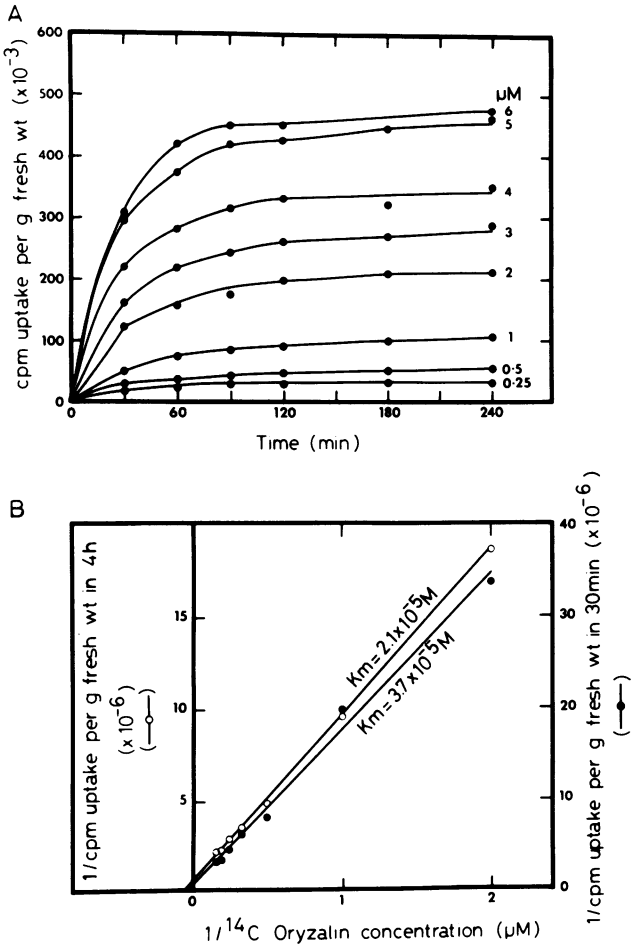

FIG. 3. Kinetics of [^{14}C]oryzalin uptake by 5-mm corn root segments. A, time course at different [^{14}C]oryzalin concentrations; B, double-reciprocal plots of the initial rates of uptake (●——●) and accumulation (cpm/g fresh weight at 4 h) (○——○).

were incubated in 10 μM [^{14}C]oryzalin for 4 h at 25 C showed a small peak of bound [^{14}C]oryzalin which appeared near the void volume of the column. This bound [^{14}C]oryzalin peak did not travel with the elution volume of the [^{3}H]colchicine-binding (microtubule subunit protein) peak of rat brain postribosomal fraction.

No evidence for [^{14}C]oryzalin binding in the postribosomal fraction of rat brain extract was obtained. In addition, DNHs did not affect the [^{3}H]colchicine binding to rat brain tubulin (Table I).

Binding in Insoluble Fraction. Differential centrifugation of root homogenates (obtained from apices incubated in [^{14}C]oryzalin for 4 h) indicated that at least 74% of the ^{14}C taken up was bound to the particulate material and was not localized in any narrow sedimentation range (Table II). However, during homogenization of root segments, some release and redistribution of bound [^{14}C]-oryzalin could have occurred.

Repeated washing of the 200,000*g* pellet released the bound [^{14}C]oryzalin (Fig. 4). About 85% of the bound ^{14}C was removed after five washes. Nonradioactive oryzalin (10 μM) in the pellet resuspension-buffer had no effect on the release of bound [^{14}C]-oryzalin.

Sonication of the homogenate did not release radioactivity bound in the insoluble fraction pelleted at 200,000*g* (Table III).

Treatment of the homogenate (from root segments incubated in [^{14}C]oryzalin) with Triton X-100 (1.25%, v/v), Nonidet P-40 (0.5%, v/v) or digitonin (0.5% w/v) solubilized the [^{14}C]oryzalin bound to the insoluble fraction (Table IV). When root homogenates were extracted with different concentrations of Nonidet P-40 and the particulate material was washed to remove the detergent before incubation with [^{14}C]oryzalin, the ^{14}C bound in the particulate material decreased with increasing detergent concentrations up to 0.3% (Fig. 5).

Boiling the homogenate had no effect on [^{14}C]oryzalin binding in the insoluble fraction (Table V).

Table I. *Effect of DNHs and Colchicine on [^{3}H]Colchicine Binding in Postribosomal Fraction of Rat Brain Extract*

One rat brain (1.9 g) was homogenized with 4.8 ml 0.1 M phosphate (NaH_2PO_4) buffer containing 0.4 M sucrose and 1 mM concentrations of EGTA $MgSO_4$ and GTP (pH 6.8). After filtering through cheesecloth, the homogenate was centrifuged at 200,000*g* for 1 h at 2 C, and 0.2 ml supernatant was incubated (90 min, 37 C) with 0.25 ml 0.1 M NaH_2PO_4 containing 1 mM concentrations of $MgSO_4$ and EGTA (pH 6.8) with or without colchicine, A-820, or oryzalin, and 50 μl 1.35 μM [^{3}H]colchicine. Radioactivity bound to tubulin in 100 μl incubation mixture was determined by DE-81 ion-exchange paper binding assay similar to that described by Weisenberg *et al.* (12).

Treatment	Radioactivity Bound to DE-81 Filter Disc
	cpm
Control	40,289
+ Colchicine, 1 mM	282
+ A-820, 1 μM	49,786
+ Oryzalin, 1 μM	41,394

Table II. *Fractionation of [^{14}C]Oryzalin Taken Up by Corn Root Segments*

Sixty root segments were incubated in 10 ml (10 μM) [^{14}C]oryzalin for 4 h and then the roots were ground in 4 ml homogenization buffer. Two ml homogenate were centrifuged (for 15 min) at successively higher velocities. The radioactivity in pellet sedimenting at each velocity was determined.

Fraction	cpm	Total cpm
g		%
0–1,000	15,259	20.2
1,000–2,500	4,445	5.9
2,500–5,000	5,111	6.8
5,000–15,000	7,541	10.0
15,000–30,000	6,761	9.0
30,000–60,000	6,463	8.6
60,000–150,000	6,952	9.2
150,000–200,000	3,376	4.5
200,000*g* supernatant	19,549	25.9
Total	75,457	

DISCUSSION

[^{14}C]Oryzalin Uptake and *in Vivo* Binding. [^{14}C]Oryzalin is taken up rapidly from the ambient medium by corn root segments and reaches a plateau in 4 h. Interestingly, 4 h is also the minimum incubation time in oryzalin required to get maximum inhibition of elongation (10). This [^{14}C]oryzalin taken up by roots is not translocated to the shoots in intact seedlings (8).

At 4 h, the overall concentration of [^{14}C]oryzalin in roots is more than 35 times higher than in the incubation medium. This accumulation is not affected by the respiratory inhibitor sodium azide and there is no metabolism of [^{14}C]oryzalin under these conditions. Therefore, most (97%) of the [^{14}C]oryzalin inside roots must (*a*) be bound to some intracellular site by a process not requiring metabolic energy and (*b*) not be accumulated as free

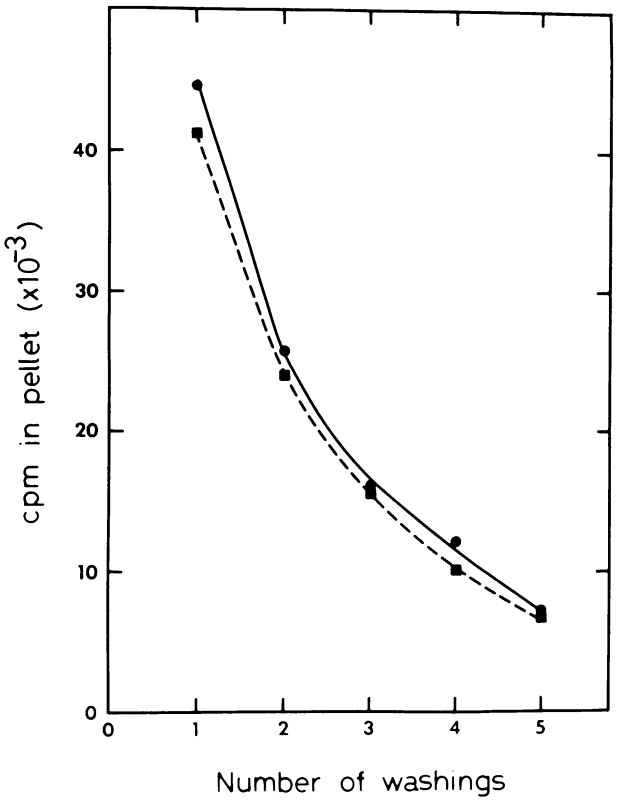

FIG. 4. Release of [^{14}C]oryzalin bound to the insoluble fraction during repeated washing of the 200,000*g* pellet by resuspension followed by centrifugation in homogenization buffer with (■– – –■) or without (●——●) 10 μM nonradioactive oryzalin. Sixty corn root apices (5 mm) were incubated in 10 ml of 10 μM [^{14}C]oryzalin and ground in 6 ml homogenization buffer. After straining through two layers of cheesecloth, the homogenate was centrifuged at 200,000*g* for 15 min. The pellets were resuspended in 2 ml homogenization buffer with or without 10 μM nonradioactive oryzalin. A 50-μl sample was taken for radioactivity measurement, and the remaining 1.95 ml were centrifuged at 200,000*g* for 15 min.

Table III. *Effect of Sonication on [^{14}C]Oryzalin Bound to Insoluble Fraction*

Six hundred corn root apices (5 mm) were incubated in 10 μM [^{14}C]-oryzalin (50 ml) for 4 h. Then, these segments (4.1 g) were ground with 10.2 ml homogenization buffer, and the homogenate was filtered through two layers of cheesecloth. Two ml of this filtrate were sonicated for 0 to 150 s (microprobe, setting 5, pulse). After each 30 s sonication, the homogenate was cooled in ice for 2 min. One ml of the sonicated homogenate from each treatment was mixed with 1 ml homogenization buffer and centrifuged at 200,000*g* for 1 h, and radioactivity in the pellets was determined.

Sonication Time	Radioactivity in 200,000*g* Pellet
s	*cpm*
0	202,974
30	191,116
60	195,813
90	192,516
120	205,549
150	204,072

oryzalin in the root cells.

The [^{14}C]oryzalin bound in root segments could be removed by washing them in incubation buffer with or without nonradioactive oryzalin, suggesting that oryzalin binding involves some reversible, low-affinity association with the binding site. At least a part of this oryzalin released on washing may cause root swelling, for

Table IV. *Effect of Membrane-solubilizing Detergents on [^{14}C]Oryzalin Bound to Insoluble Fraction*

Homogenate obtained from 60 root apices as described in Table II was incubated with detergents for 15 min at 4 C and then centrifuged at 200,000*g* for 1 h.

Treatment	Radioactivity		
	In 200,000*g* Pellet	In 200,000*g* Supernatant	Total
	cpm		
Control (buffer only)	19,648	12,430	32,078
Triton X-100 (1.25% v/v)	1,317	35,260	36,577
Nonidet P-40 (0.5% v/v)	1,888	35,020	36,908
Digitonin (0.5% w/v)	5,200	29,170	34,370

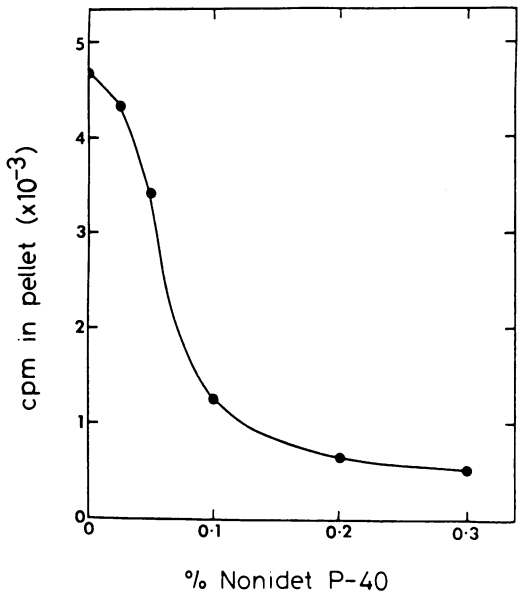

FIG. 5. Effect of Nonidet P-40 on [^{14}C]oryzalin binding in the insoluble fraction. After 3 h preincubation, 150 corn root apices (1.1 g) were homogenized with 14 ml homogenization buffer (containing 5 mM cysteine) and the homogenate was filtered through two layers of cheesecloth. The filtrate (1.8 ml) was extracted with Nonidet P-40 (final concentrations, 0.025, 0.05, 0.1, 0.2, and 0.3% v/v) at 4 C for 20 min and centrifuged at 200,000*g* for 1 h. The pellets were washed (5 times) with 0.5 ml homogenization buffer to remove the Nonidet P-40 and resuspended in 3 ml homogenization buffer. Two ml of the latter suspension were incubated with 25 μl 80 μM [^{14}C]oryzalin (final concentration, 1 μM) at 25 C for 20 min. The incubation mixture then was centrifuged at 200,000*g* and the radioactivity in the pellets was determined.

transfer of intact roots to water after 4 h incubation in 1 μM oryzalin does not produce maximal swelling; longer exposure to oryzalin (up to 72 h) produces more swelling (10). Because of the low solubility of oryzalin (lower than the K_m of binding), competition studies with nonradioactive oryzalin and its analogs could not be done.

Binding in Soluble Fraction. To determine if [^{14}C]oryzalin interacts with microtubule subunit protein, its binding in the postribosomal fraction of corn root and rat brain [a rich source of microtubule subunit protein tubulin (12)] extracts was studied. Although Hess and Bayer (4) have reported an interaction of [^{14}C]trifluralin (a DNH) with a tubulin-like protein isolated from *Chlamydomonas* flagella, we were unable to demonstrate DNH binding or even substantial colchicine binding to any tubulin-like

Table V. *[^{14}C]Oryzalin Binding in Insoluble Fractions of Boiled and Unboiled Homogenates*

Five-mm root apices (150, 1.1 g), incubated in 50 ml incubation buffer, were ground in 4.8 ml homogenization buffer without cysteine and phenylmethylsulfonyl fluoride. After filtering through cheesecloth, 2 ml homogenate were stored on ice and 2 ml were kept in boiling water bath for 15 min. One-half ml of the boiled or unboiled homogenate was added to 1.5 ml homogenization buffer containing 1 μM [^{14}C]oryzalin and incubated at 25 C for 30 min. The incubation mixture was centrifuged at 200,000*g* for 1 h at 2 C and radioactivity in the pellets was determined.

Replicate	Radioactivity in Pellet	
	Unboiled Homogenate	Boiled Homogenate
	cpm	
I	4,038	4,827
II	4,023	4,465
III	3,667	4,023
Mean ± SE	3,909 ± 121	4,438 ± 232

protein in corn root extracts. This could, of course, be due to a variety of technical problems; however, a procedural check using rat brain extracts did show colchicine binding where it was expected. The binding of colchicine to rat brain tubulin is not affected by DNHs, and [^{14}C]oryzalin does not bind to rat brain tubulin. Bartels and Hilton (2) also reported that trifluralin neither binds to porcine brain tubulin nor affects the *in vitro* polymerization of tubulin into microtubules. Thus, DNHs do not seem to interact with vertebrate tubulin.

Binding in Insoluble Fraction. To determine where most of the [^{14}C]oryzalin taken up by roots is bound, binding to insoluble material was studied. Differential centrifugation of the homogenate, obtained from root segments incubated for 4 h in [^{14}C]-oryzalin, indicates that most of the [^{14}C]oryzalin is bound to the particulate material but not distinctly localized in any particular sedimentation range. Corresponding to the reversibility of uptake and binding in root segments, [^{14}C]oryzalin bound in the 200,000*g* pellet could be released by repeated washing (resuspension followed by centrifugation) in the homogenization buffer; thus, [^{14}C]oryzalin is associated with particulate material by some weak, reversible bonding.

The oryzalin bound in the insoluble fraction could be solubilized with mild membrane detergents, such as Nonidet P-40 (0.5%, v/v), Triton X-100 (1.25%, v/v), and digitonin (0.5%, w/v). The release of ^{14}C by detergent treatment is apparently due to solubilization (or inactivation) of the oryzalin-binding site, for the treatment of root extracts with Nonidet P-40 followed by washing of detergent prior to incubation with [^{14}C]oryzalin decreases the subsequent [^{14}C]oryzalin binding in the insoluble fraction. The effect of membrane detergents also suggests that [^{14}C]oryzalin binds to some site associated with cellular membranes. Sonication does not release any bound oryzalin from the insoluble fraction. Therefore, [^{14}C]oryzalin is not bound to any site (microtubule subunit or any other protein) loosely attached to membranes or held within membranous vesicles. Boiling the homogenate, which would have denatured most proteins, had no effect on [^{14}C]oryzalin binding, suggesting that proteins are not involved in this binding. It appears from these results that [^{14}C]oryzalin binds to (or is partitioned into) the lipid (and not protein) component of cellular membranes. Indeed, the lipophilic character of the DNHs supports this idea. Preliminary investigations on the physiological significance of this interaction of oryzalin with membranes suggest that this binding affects the permeability properties of the membrane(s) (7) and may be involved in the differential species resistance to oryzalin (11). These results will be published elsewhere.

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