Metabolism of 3',4'-Dichloropropionanilide: 3,4-Dichloroaniline–Lignin Complex in Rice Plants

Abstract. *Rice plants* (Oryza sativa *L. var. Bluebonnet 50*) *metabolize the herbicide 3',4'-dichloropropionanilide to 3,4-dichloroaniline, which in turn conjugates with carbohydrates. Soluble aniline-carbohydrate complexes account for only a small fraction of the hydrolyzed 3',4'-dichloropropionanilide. The major portion of the 3,4-dichloroaniline moiety is found complexed with polymeric cell constituents, mainly lignin. The aniline is lignin-bound as 3,4-dichloroaniline and not as 3',4'-dichloropropionanilide.*

The selective postemergence herbicide 3',4'-dichloropropionanilide (DPA) has been used extensively during the past several years for weed control in rice (*1*). Investigations on the mechanism of selective action of DPA in rice (*Oryza sativa* L. var. Bluebonnet 50) (DPA tolerant) and barnyard grass (*Echinochloa crusgalli*) (DPA susceptible) have shown that rice leaves rapidly hydrolyze DPA to give free 3,4-dichloroaniline (DCA), but hydrolytic metabolism does not occur readily in barnyard grass (*2*). Recently Still (*3*) recovered free DCA and three metabolites containing DCA from DPA-treated rice plants. However, 14 days after treatment the free aniline and metabolites accounted for only 10 percent of the applied DPA, even though complete hydrolysis had occurred. The remaining aniline moiety was not recovered and was assumed to have ended up in unknown intermediates. This report confirms the presence of DCA metabolic complexes in rice treated with C^{14}-ring-labeled DPA. It also describes investigations which show that the major portion of DCA resulting from rice leaf hydrolysis of DPA is complexed with polymeric cell constituents. The highest percentage of DCA was complexed with lignin. Most, if not all, of the aromatic portion of DPA can be accounted for as DCA in rice leaves.

Table 1. Radioactivity recovered in ring-labeled-C^{14} DPA-treated rice plants. Two-week-old plant leaves each received 30 μg of ring-labeled-C^{14} DPA in 10 μl of acetone. Liquid samples were determined by the liquid scintillation technique. Ground residue was determined by combustion. Results are presented as percent of total C^{14} radioactivity applied. Fifty seedlings were used in each sample.

Days after treatment	Radioactivity recovered (%)	
	Methanol extract	Plant residue
1	72.2	5.0
3	61.6	10.8
7	36.0	25.7
14	13.7	33.5
21	7.7	38.4

Homogenates of aerial parts of greenhouse-grown plants treated with ring-labeled-C^{14} DPA (specific activity, 1.01 mc/g) were extracted with methanol and the fractions were analyzed for radioactivity. Analyses made on plants 1, 3, 7, 14, and 21 days after treatment with DPA showed (Table 1) that the methanol-soluble C^{14} fraction decreased and the insoluble residue C^{14} fraction increased with time. The methanol-soluble fraction contained unhydrolyzed DPA, DCA, and four metabolites all of which decreased with time. No attempt was made to achieve a materials balance in the experiment, and the reason for C^{14} loss is not known. Radioactivity was found in roots, but less than the amount which disappeared.

Still (*3*) found three DCA metabolites, one identified as *N*-(3,4-dichlorophenyl)-glucosylamine in the methanol extract of rice plants treated with DPA in the solution culture, in contrast to the four metabolites found in our studies. Since DPA is a postemergence herbicide, we preferred foliar application for our metabolic studies and this may account for the difference between Still's and our results. We have designated our metabolites as M-1, M-2, M-3, and M-4 by decreasing R_F values (Fig. 1).

Our metabolite M-1 was positively identified as *N*-(3,4-dichlorophenyl)-glucosylamine by reverse radioisotope dilution and infrared spectra [identical to synthetic *N*-(3,4-dichlorophenyl)-glucosylamine, chemically synthesized by acid-catalyzed condensation of glucose with DCA (*4*)]. Metabolite M-2 was identified as a DCA saccharide conjugate which contained glucose, xylose, and fructose but was not further identified. Metabolite M-3 was not stable and readily decomposed to metabolite M-1. Metabolite M-4 was a DCA sugar derivative, but the identity of the sugars was not determined. When we treated rice plants with C^{14}-labeled DCA the above four metabolites were also found. Our results also confirm the finding of Still (*3*) in that 3,3',4,4'-tetrachloroazobenzene was not recovered in any of our rice studies.

Because there was a large portion of C^{14} radioactivity present in the plant residue after methanol extraction, we combined the plant residue samples (Table 1) and fractionated these into three fractions, namely, cellulose-hemicellulose, lignin, and water filtrate (used for precipitating lignin), by using the method of Pepper and Wood (*5*). The distribution of the radioactivity in cellulose-hemicellulose, lignin, and water filtrate was 25.7, 42.4, and 28.6 percent, respectively. Thus, the lignin fraction contained the greatest amount of C^{14} even though lignin represents only 4.8 percent of the composition of the young rice plants. The isolated radioactive lignin was analyzed for DCA by hydrolysis-steam distillation-extraction (*6*) into hexane. The hexane extract was chromatographed on a silica gel thin-layer plate, developed in acetone : benzene (15 : 85, by volume), and DCA was the only C^{14} compound found in the lignin hydrolysate. Similarly, only C^{14} DCA was recovered from the cellulose-hemicellulose and water filtrate fractions.

Since the DCA was recovered from lignin following alkaline hydrolysis, we did not know whether the aniline was complexed as DCA or DPA. Through use of C^{14}-carbonyl-labeled DPA it was possible to establish unequivocally that

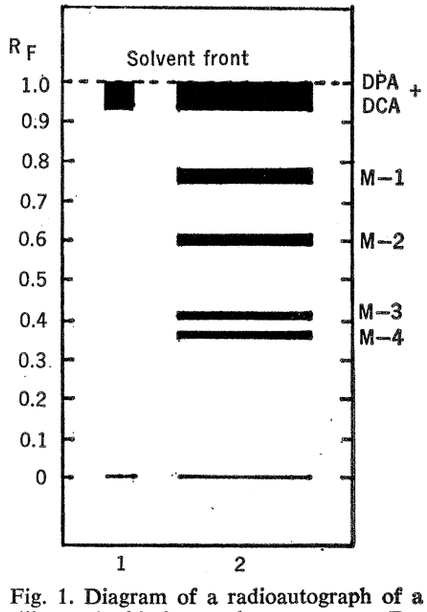

Fig. 1. Diagram of a radioautograph of a silica gel thin-layer chromatogram. Developed with *n*-butanol : pyridine : water (6 : 4 : 3). (1) Standard mixture C^{14} DPA and C^{14} DCA. (2) Methanol extract from C^{14}-ring-labeled DPA-treated rice leaves (3-day treatment).

DCA and not DPA is complexed with lignin. One group of 2-week-old, greenhouse-grown rice plants was treated with ring-labeled-C^{14} DPA and a comparable group was treated with carbonyl-labeled-C^{14} DPA (specific activity 0.85 mc/g). Lignin was isolated from the two groups of plants 3 weeks after treatment. As in the previous experiment, a high percentage of C^{14} was found in the lignin extracted from plants treated with ring-labeled DPA. The amount recovered was 80-fold greater than that found in the lignin extracted from carbonyl-labeled DPA-treated plants. This clearly established that DCA and not DPA was the compound bound to lignin. Lignin was also isolated from mature field-grown rice straw from groups of plants treated with C^{14}-ring- and C^{14}-carbonyl-labeled DPA. Again, lignin from plants treated with ring-labeled DPA contained the greater amount of radioactivity. The differences were not so great as found in the greenhouse experiment. This, however, is not unexpected, since C^{14} from the propionic acid moiety unquestionably would be available to the organic acid pool and subsequently would be incorporated into lignin.

Our studies demonstrate that most of the aniline moiety from DPA-treated rice leaves is complexed as DCA with soluble carbohydrates and polymeric cell constituents. We were not able to isolate any additional C^{14}-labeled compounds from rice treated with C^{14}-ring-labeled DPA. If others are present, they constitute a small fraction when compared with the amount of C^{14} DCA recovered.

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Mitotic Apparatus: The Selective Extraction of Protein with Mild Acid

Abstract. *The treatment of isolated mitotic apparatus with mild (pH 3) hydrochloric acid results in the extraction of less than 10 percent of its protein, accompanied by the selective morphological disappearance of the microtubules. The same extraction can be shown to dissolve outer doublet microtubules from sperm flagella. A protein with points of similarity to the flagellar microtubule protein is the major component of the extract from mitotic apparatus.*

Since Mazia and Dan (*1*) first isolated the mitotic apparatus, efforts have been made to extract from it molecules which are functional in mitosis (*1–7*). Recent work (*5–7*) has sought to equate various proteins extracted from mitotic apparatus with protein from microtubules, which are the ultrastructural equivalents of the spindle fibers. The mitotic apparatus has been treated with various media which simultaneously cause the disappearance of the microtubules and the solubilization of protein components. In these studies, 40 to 70 percent of the protein from mitotic apparatus has been solubilized under conditions leading to the disappearance of the microtubules. We now describe a method for making microtubules disappear selectively from mitotic apparatus, with extraction of less than 10 percent of the protein; a protein with properties reasonable for protein from microtubules is recovered from the extract. The extraction method, treatment with mild HCl (*p*H 3), is that used to reduce bacterial flagella to their monomer protein, flagellin (*8*). Our use of the method is based on the consideration that bacterial flagella might be a structure of the microtubule family, with chemical properties resembling those of microtubules in higher organisms.

Eggs of *Strongylocentrotus purpuratus* were fertilized and allowed to develop at 15° to 17°C. Mitotic apparatus was isolated from eggs at metaphase of the first cleavage division with molar hexylene glycol at *p*H 6.4 (*9*). Further work was carried out at 1° to 4°C. The isolated unit was washed, by centrifugation and resuspension, four times in isolation medium and twice in 0.01*M* phosphate buffer (*p*H 5.5) to remove hexylene glycol. Figure 1A indicates the appearance of mitotic apparatus at this stage of treatment. The mitotic appara-

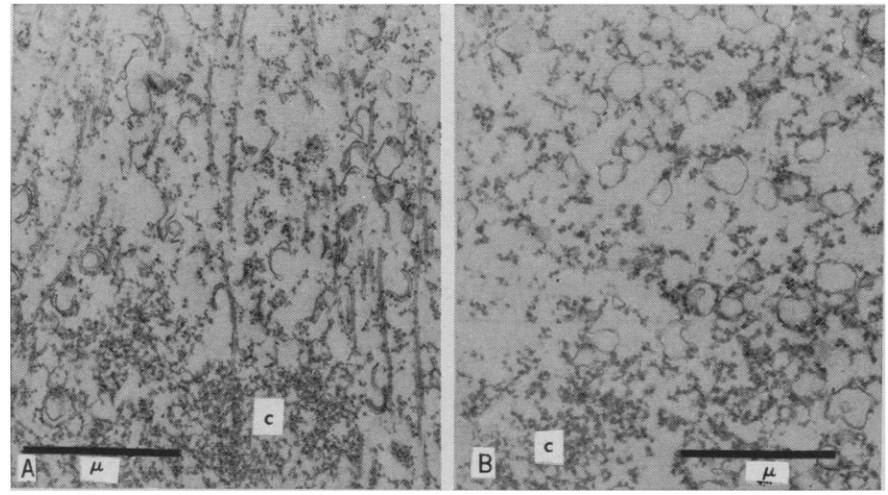

Fig. 1. (A) Isolated mitotic apparatus before acid extraction, fixed in cold 2 percent osmium tetroxide in isotonic acetate buffer (*p*H 6.1) and embedded in Epon 812. The section shown is approximately a sagittal section of mitotic apparatus as determined by previous cutting of thick sections (4 μm) and examination of these by phase microscopy. Microtubules are seen leading from the chromosomes (*c*). Ribosomes and flattened vesicles are present. An amorphous material in which ribosomes and vesicles appear to be embedded is dimly visible. Section stained 30 seconds with 0.1 percent lead citrate. (B) Appearance of isolated mitotic apparatus after 2 hours of acid extraction. Fixation and further processing as in Fig. 1A. No microtubules are visible. Ribosomes, chromosomes (*c*), and vesicles retain their morphological integrity. Ribosomes and chromosomal material appear somewhat swollen and diffuse. Vesicles have partially rounded up.