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Modeling of the Covalent Attachment of Chloroaniline Residues to Quinoidal Sites of Soil Humus[1]

Adesh Saxena and Richard Bartha

Department of Biochemistry and Microbiology, Cook College, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, NJ 08903

The microbial metabolism of various phenylamide herbicides in soil releases substituted anilines (CRIPPS & ROBERTS 1979), that in turn become inextractable by organic solvents (CHISAKA & KEARNEY 1970; BARTHA 1971). Investigations performed on 3,4-dichloroaniline (DCA) revealed that immobilization occurs by rapid covalent binding of DCA to soil humus (BARTHA 1971; HSU & BARTHA 1974a). DCA is not structurally altered by the binding process and can be released unchanged by hydrolytic treatment (YOU & BARTHA 1982b), by exchange with other amines (PARRIS 1980; SAXENA & BARTHA 1983a) or by biodegradation of soil humus (HSU & BARTHA 1974b SAXENA & BARTHA 1983b). Apparently, through this latter mechanism (STILL et al. 1980), DCA was found to occur as low-level contaminant in all tested market rice samples (STILL & MANSAGER 1969).

The chemical identity of the receptor sites for DCA on humic acid (HA) could not be identified by IR spectrometry (BARTHA & HSU 1976). Therefore, the binding mechanism was investigated by indirect modeling approaches (HSU & BARTHA 1974a, 1976; PARRIS 1980), but until recently, even the exact structure of the model complexes remained unidentified. YOU et al. (1982) synthesized a chemically defined model adduct N-(3,4-dichlorophenyl)-3-hydroxy-6-methyl-p-benzoquinonimide by reacting DCA with 4-methyl-catechol, but the properties of the model differed substantially from those of HA-DCA complexes. Based on kinetic and exchange considerations, PARRIS (1980) recently proposed a plausible binding scheme for anilines to quinoidal sites of HA. Prompted by this report and our own preliminary investigations on DCA binding to benzoquinone (HSU & BARTHA 1976), we synthesized a model adduct from DCA and 2-methyl-benzo-1,4-quinone (toluquinone), identified its chemical structure, and compared its behavior to that of HA-DCA complexes.

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EXPERIMENTAL

Chemicals. Uniformly-labeled DCA (specific activity 61 uci/mg) (Amersham-Searle, Des Plaines, IL) had a radiochemical purity, as determined by TLC (Silica gel, toluene:ethyl acetate 9:1) of 97%. Unlabeled DCA and 4-chloroaniline (Aldrich, Milwaukee, WI) were purified by recrystallization from petroleum ether to a melting point of 71°C (STECHER 1968). Aniline, 4-methylaniline, and N-methylaniline (Aldrich) were purified by distillation. Toluquinone(TQ) was purchased from Eastman Kodak (Rochester, NY). Solvents were of high-purity grade or were distilled prior to use. Complexes of humic acid (HA) and radiolabeled DCA were prepared as described earlier (SAXENA & BARTHA 1983b) and had a specific activity of 760 dpm/mg.

Analytical Procedures. TLC separations were performed on 250-µm thick commercially coated silica gel G plates (Fisher, Pittsburgh, PA). GC analysis was performed on a Hewlett-Packard Model 5700A instrument with dual 0.6 x 180 cm stainless steel columns packed with 5% UCW-98 on Chromosorb W. Carrier: N_2, 30 mL/min, oven 225°C isothermal, dual FID detectors 300°C. Mass spectra were recorded on a Hewlett-Packard Model 5985 instrument using a direct insertion probe at 70 eV. ^1H NMR spectra were recorded on a Varian CFT-20 instrument using deuterated dichloromethane as solvent. IR spectra were recorded in KBr pellet in a Beckman (Fullerton, CA) Acculab 2 instrument. UV and visible spectra were recorded in methanol solution on a Bausch & Lomb Spectronic 2000 instrument. Radioactivity was measured by liquid scinitillation counting in Aquasol (New England Nuclear, Boston, MA) and a Beckman LS230 counter. $^{14}CO_2$ was trapped and counted in oxifluor (NEN). Counts were corrected for background and quenching using the external standard ratio method. Melting points were determined using a Fisher-Johns apparatus and were uncorrected.

Preparation and Purification of the Model Adduct. TQ was reacted with equimolar amounts of unlabeled or radiolabeled DCA in 0.2 M phosphate buffer (pH 6.0) at 28°C in the dark. TQ (195 mg) and DCA (260 mg) were dissolved in 5 mL of acetone, immediately followed by 95 mL 0.2 M pH 6.0 phosphate buffer, and the mixture was incubated in the dark on a rotary shaker and under an air atmosphere for 3 days. The major (GC retention 2 min, TLC R_f = 0.12 with benzene) and the minor (GC retention 2.2 min, TLC R_f 0.07 with benzene) products of the reaction were collected by filtration. The product mixture was dissolved in benzene, and its two components were separated on a 2 x 39 cm glass column packed with silica gel (Woelm, activity grade III) and eluted with benzene. The major product eluted before the minor one. The major product was further purified on two successive TLC plates, the first developed with benzene (R_f 0.12) and the second with benzene:acetone (19:1, R_f 0.57). The major product, hereafter referred to as TQ-DCA adduct, was scraped from the second TLC plate, was dissolved in

chloroform,and was rechromatographed on a silica gel column using chloroform for elution. The now homogeneous TQ-DCA adduct was concentrated and dried in a rotary evaporator under vacuum.

Acidic and Alkaline Hydrolysis. The susceptibilities of the TQ-DCA adduct and of the HA-DCA complex to chemical hydrolysis were compared as described by YOU et al. (1982). The specific activities of the TQ-DCA adduct and the HA-DCA complex were 8,591 and 760 dpm/mg, respectively; 1 mg TQ-DCA adduct and 50 mg HA-DCA complex were used in the hydrolysis experiments.

Exchangeability. 0.2 mg radiolabeled TQ-DCA adduct in 0.01 mL acetone was added to 0.5 mL methanol in each of six 1-mL serum vials. Two mg each of aniline, 4-methylaniline, 4-chloroaniline, and N-methylaniline were added individually as displacing agents to 4 of the vials. The fifth vial received 0.1 mL of 12 M urea, the sixth vial remained untreated as a control. All vials were sealed with Teflon-lined aluminum caps and were incubated with agitation at 25°C in the dark for 3 days. At this time,dilute aqueous HCl was added to prevent DCA loss by evaporation, and the methanol was evaporated under an N_2-stream. The DCA was extracted from the aqueous solution into ethyl acetate. After TLC separations, 14-C DCA was quantified by liquid scintillation counting (LSC).

Biodegradation in Soil. To freshly collected and sieved samples of Nixon sandy loam (HSU & BARTHA 1974a), equivalent to 10 g by oven-dry weight,1.7 mg radiolabeled TQ-DCA adduct dissolved in 0.1 mL acetone was added and was thoroughly mixed. The acetone was allowed to evaporate, and the treated soil was transferred to modified micro-fernbach flasks (MARINUCCI & BARTHA 1979), containing sufficient water to adjust soil moisture to 60% of capacity. In some flasks, 100 ppm aniline on soil dry weight basis was added to this water to stimulate mineralization of the TQ-DCA adduct (YOU & BARTHA 1982a). A control poisoned with 1% $HgCl_2$ was included. All flasks were incubated at 28°C in the dark and periodically flushed with air through traps (MARINUCCI & BARTHA 1979). Trapped $^{14}CO_2$ was quantified by LSC.

RESULTS

Characterization of the TQ-DCA Adduct. Besides the residual reactants TQ and DCA, the reaction mixture contained a major (40% yield) and a minor (14% yield) product. Both crystallized as violet needles and, as indicated by their spectra, were isomers. Only the major TQ-DCA adduct that melted at 193°C and was readily soluble in acetone, benzene, chloroform, diethyl ether, ethyl acetate, methanol and dichloromethane, poorly soluble in CCl_4, dioxane, DMSO, hexane and water, was characterized and used in the subsequent experiments.

In the UV and visible range, the TQ-DCA adduct had a (MeOH) μ_{max} at 269 and 485 nm, and both maxima shifted with pH. The mass spectrum of the TQ-DCA adduct showed peaks at m/e (rel. intensity) 281/283/285 (100/66/12), 266/268 (12/7), 252/254/256 (22/16/4), 246/248 (29/9), 238/240 (12/8) 224/226 (10/8), 218/220 (13/5), 212/214/216 (47/32/6) 185/187 (18/12). The IR spectrum (KBr pellet) showed bands at 3220 (N-H stretch), and 16.65, 16.30 (C=O) cm^{-1}.

The ^{1}H-NMR spectrum (deuterated dichloromethane) showed signals at δ7.49 (S, 1, N-H), 7.36 (d, 1, J_o=4.0 Hz) 7.29 (d, 1, J_m=2.0 Hz), 7.0/17.12 (dd, 1, J_o=9.0 Hz), 6.55 (q, 1. $^4J_{CH_3-H}$=3.2 Hz), 6.08 (S, 1), 2.06 (d, 3, $^4J_{CH_3-H}$=2.0 Hz).

By comparison of the above data to spectra of TQ and DCA, the adduct was identified as shown in Fig. 1.

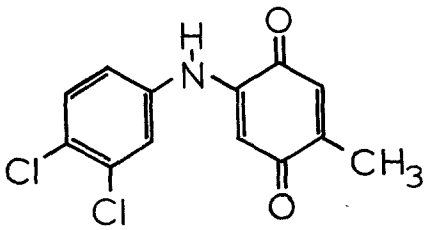

Figure 1. Chemical structure of the TQ-DCA adduct 2-(3,4-dichloroaniline)-5-methyl-benzo-1,4-quinone.

Acidic and Alkaline Hydrolysis. The data in Table 1 show excellent agreement between the TQ-DCA adduct and HA-DCA complex in sensitivity to acid hydrolysis. The sensitivity of the TQ-DCA adduct to alkaline hydrolysis was, however, lower than that of the HA-DCA complex. DCA itself is stable under the conditions of hydrolysis (YOU et al. 1982).

Table 1. Comparative release of ^{14}C-DCA from TQ-DCA adduct and HA-DCA complex.

	Hydrolytic Release of ^{14}C DCA (%)[1] by	
Parent Compound	70% H_2SO_4	50% NaOH
TQ-DCA adduct	66.0 (± 6.0)	31.0 (± 4.5)
HA-DCA complex	64.0 (± 3.0)	72.0 (± 2.5)

[1]Average of triplicate determinations; standard deviation in parenthesis.

Exchangeability. Intact DCA was liberated from TQ-DCA complexes by exchange with aromatic amines and urea. The effectiveness of the individual displacing agents correlated with their basicity (Table 2).

Table 2. Displacement of ^{14}C-DCA from TQ-DCA adduct by aromatic amines and urea.

Displacing Agent	^{14}C-DCA Displaced (%)[1]
Aniline	50.0 (± 7.2)
4-methylaniline	39.0 (± 2.5)
N-methylaniline	5.0 (± 1.2)
4-chloroaniline	5.0 (± 2.5)
Urea	11.0 (± 1.8)

[1]Average of triplicate determinations; standard deviation in parenthesis.

Similar displacement from HA-DCA complexes was reported by PARRIS (1980) and SAXENA & BARTHA (1983b).

Biodegradation in Soil. $^{14}CO_2$ release from TQ-DCA adduct and from HA-DCA complex incubated in soil is shown in Table 3.

Table 3. Comparative biodegradation of radiolabeled TQ-DCA adduct and HA-DCA complex in soil.

Parent Compound	Aniline	$^{14}CO_2$-produced (% per day)[1]
TQ-DCA	-	0.17 (± 0.06)
TQ-DCA	+	0.65 (± 0.14)
HA-DCA	-	0.09 (± 0.02)
HA-DCA	+	0.37 (± 0.09)

[1]Average of triplicate determinations; standard deviation in parenthesis. Biodegradation rates for HA-DCA complexes were calculated from data by YOU & BARTHA (1982a).

Both compounds were biodegradable at rather slow rates, HA-DCA complexes being somewhat more recalcitrant. Aniline stimulated the biodegradation of both compounds to a similar degree, increasing both rates approximately four-fold.

DISCUSSION

An examination of the chemical structure of the model compounds showed it to be a 1:1 adduct of TQ and DCA. Its formation is analogous to the oxidative binding of anilines to benzoquinone or HA, as postulated by PARRIS (1980). This binding mechanism is also supported by the fact that aniline and 4-methylaniline displaced as much as 50% and 39% of the bound DCA from the model compound. Aniline and 4-methylaniline that are more basic than 4-chloroaniline and N-methylaniline were found to be more effective in displacing DCA. Urea, though not an aniline, is a weakly basic organic amine that displaced DCA from the TQ-DCA adduct as well as from HA-DCA complexes (SAXENA, unpublished observation). A corresponding relationship between aniline basicity and effectiveness in displacing DCA from HA-DCA complexes was reported by PARRIS (1980) and SAXENA & BARTHA (1983a).

Acid and alkaline hydrolysis liberated intact DCA from TQ-DCA as well as from HA-DCA, but the sensitivity of the TQ-DCA adduct to alkaline hydrolysis was lower in comparison to the HA-DCA complex. Considering the vast difference in complexity between the TQ-DCA adduct and the HA-DCA complex, and the fact that the latter is not a homogeneous chemical compound, some quantitative differences between the behavior of the HA-DCA complex and the monomeric model are not unexpected. Quinoidal sites of HA are obviously not the only bonding possibility for DCA, though they appear to be the most stable ones and the role of such stable bonds in DCA binding appears to increase with incubation time (HSU & BARTHA 1976, YOU & BARTHA 1982b).

The biodegradation experiments are generally supportive of the proposition that the quinoidal sites of HA are the predominant binding sites for DCA. The biodegradation rate of the TQ-DCA adduct is of the same order of magnitude as that of the HA-DCA complex though, not surprisingly, the monomeric model is degraded somewhat faster. Again, the complexity of the HA polymer and the possible inaccessibility of some of the bound DCA to microorganisms appears to offer an adequate explanation for the quantitative difference. It should be also noted here that the TQ-DCA adduct models only the first complete cycle of DCA attachment to HA. As pointed out by PARRIS (1980), further addition and oxidation reactions may lock aniline residues increasingly strongly into the humic polymer.

The fact that biodegradation of TQ-DCA adduct and of HA-DCA complex are stimulated by addition of aniline to the same fourfold extent strongly supports the validity of the TQ-DCA model. It is useful to contrast the findings described in this paper with an earlier evaluation of a model that envisioned the attachment of DCA to phenolic sites of HA. N-(3,4-Dichlorophenyl)-3-hydroxy-6-methyl-p-benzoquinonimide was formed by the 1:1

addition of 4-methylcatechol (MC) and DCA and is referred to as MC-DCA adduct (YOU et al. 1982). This compound resisted alkaline hydrolysis completely, had a biodegradation rate that was two orders of magnitude lower (0.005% day^{-1}) than that of the HA-DCA complex, and this low biodegradation rate was not enhanced by an addition of aniline. For these reasons, the MC-DCA adduct was rejected as a valid model for DCA attachment to HA.

Within the limitations inherent to all models, we feel that the behavior of the TQ-DCA adduct is very similar to that of HA-DCA complexes. Our findings support the theory of PARRIS (1980) who first suggested that quin oidal moieties of humic polymers are the principal sites for the covalent attachment of pesticide-derived chloroaniline residues.

REFERENCES

BARTHA, R.: J. Agric. Food Chem. 19:385 (1971).

BARTHA, R., and T.-S. HSU: pp. 258-271. In: D. D. Kaufman, G. G. Still, G. D. Paulson, S. K. Bandal (eds.). Bound and Conjugated Pesticide Residues. A.C.S. Symposium Series No. 29, American Chemical Society, Washington, D.C. 1976.

CHISAKA, M. and P. C. KEARNEY: J. Agric. Food Chem. 18:854 (1970).

CRIPPS, R. E. and T. R. ROBERTS: pp. 667-730. In: I. R. Hill and S. J. L. Wright (eds.). Pesticide Microbiology. Academic Press, New York. 1978.

HSU, T.-S. and R. BARTHA: Soil Sci. 116:444 (1974a).

HSU, T.-S. and R. BARTHA: Soil. Sci. 118:213 (1974b).

HSU, T.-S. and R. BARTHA: J. Agric. Food Chem. 24:118 (1976).

MARINUCCI, A. C. and R. BARTHA: Appl. Environ. Microbiol. 38:1020. (1979).

PARRIS, G. E.: Environ. Sci. Technol. 14:1099 (1980).

SAXENA, A. and R. BARTHA: Soil Sci. in press (1983a).

SAXENA, A. and R. BARTHA: Soil Biol. Biochem. in press (1983b).

STECHER, P. G. (ed.): p. 350. The Merck Index, 8th Edition, Merck and Co., Inc., Rahway, NJ 1968.

STILL, C. C., T.-S. HSU, R. BARTHA: Bull. Environ. Contam. Toxicol. 24:550 (1980).

STILL, G. G. and E. R. MANSAGER: Weed Res. 9:218 (1969).

YOU, I.-S. and R. BARTHA: Appl. Environ. Microbiol. 44:678 (1982a).

YOU, I.-S. and R. BARTHA: J. Agric. Food Chem. 30:1143. (1982b).

YOU, I.-S., R. A. JONES, R. BARTHA: Bull. Environ. Contam. Toxicol. 29:476 (1982).

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