Azobenzene Residues from Aniline-based Herbicides; Evidence for Labile Intermediates

by L. M. BORDELEAU and R. BARTHA
Department of Biochemistry and Microbiology,
Rutgers—The State University,
New Brunswick, New Jersey

The biochemical synthesis of relatively persistent azobenzene residues from the aromatic moieties of certain aniline-based herbicides in soil (1, 2, 3, 4, 5) represents an intriguing and potentially important facet of environmental contamination. This first paper of a series on the mechanism of this "reverse degradation" reports the detection and tentative identification of labile intermediates. As peroxidatic reactions have been implicated in the transformation of anilines (6), experiments in soil were supplemented by experiments in a peroxidase reaction mixture.

Materials and Methods

Substituted anilines (Aldridge Chem. Co.) were purified by distillation or by recrystallization from ligroin. Symmetric azobenzenes were synthesized as reported elsewhere (7). Asymmetric azobenzene standards were obtained by letting appropriately substituted anilines and nitrosobenzenes react overnight in glacial acetic acid (8). 4-Chlorophenylhydroxylamine was synthesized from 4-chloronitrobenzene by reduction with zinc powder in the presence of ammonium chloride (9). Equal parts of ethanol and water served as solvent. 4-Chloronitrosobenzene was prepared by sodium dichromate oxidation of 4-chlorophenylhydroxylamine in acid medium (10). The product was purified by distillation and recrystallized from an acetone-water mixture. 4,4'-Dichloroazoxybenzene was obtained by reacting 4-chlorophenylhydroxylamine with 4-chloronitrosobenzene in alkaline acetone solution (10). The purity of all compounds was established by gas-liquid and by thin layer chromatography. Anilines and suspected intermediates, alone or in combination, were incubated in Nixon sandy loam for 5 days or in a horseradish peroxidase reaction mixture for 2 hours, extracted, and analyzed by gas- and thin layer chromatography as described earlier (6). To improve the separation of azobenzenes, the liquid phase of the column packing was increased from 5-10%, and ligroin (60-70^{o}C) was used instead of benzene for developing the thin layer plates.

Results and Discussion

Formation of a molecule of azobenzene from two molecules of aniline involves the removal of the total of 4 electrons. Experience shows, that biochemical reactions eliminate electrons stepwise. This theoretical consideration seemed to justify a search for labile intermediates in the course of azobenzene formation.

Bulletin of Environmental Contamination & Toxicology,
Vol. 5, No. 1, 1970, published by Springer-Verlag New York, Inc.

TABLE I

Retention times and R_f-values of some substituted azobenzenes

Azobenzenes	Retention (sec)	R_f
4,4'-Dimethylazobenzene	120	0.30
4-Chloro-4'-methylazobenzene	144	0.45
4,4'-Dichloroazobenzene	174	0.60
4,3',5'-Trichloroazobenzene	248	0.81
4,3',4'-Trichloroazobenzene	288	0.68
3,3',5,5'- Tetrachloroazobenzene	348	0.90
3,3',4,4'- Tetrachloroazobenzene	468	0.66

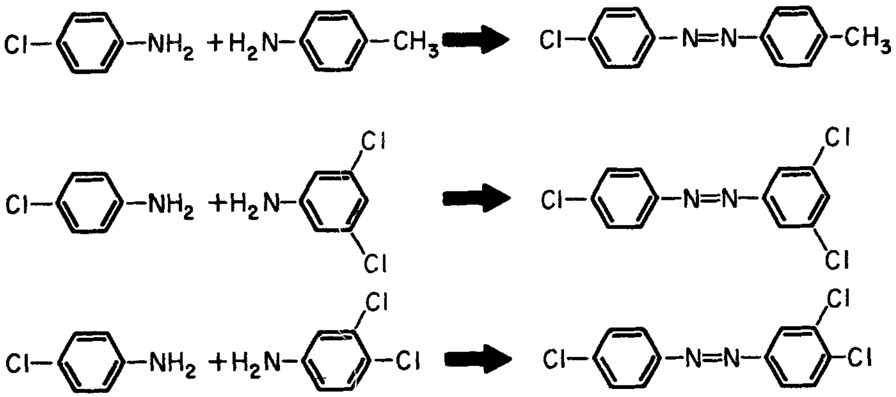

Figure 1. Formation os asymmetric azobenzenes from pairs of differently substituted anilines in soil and in peroxidase reaction mixture.

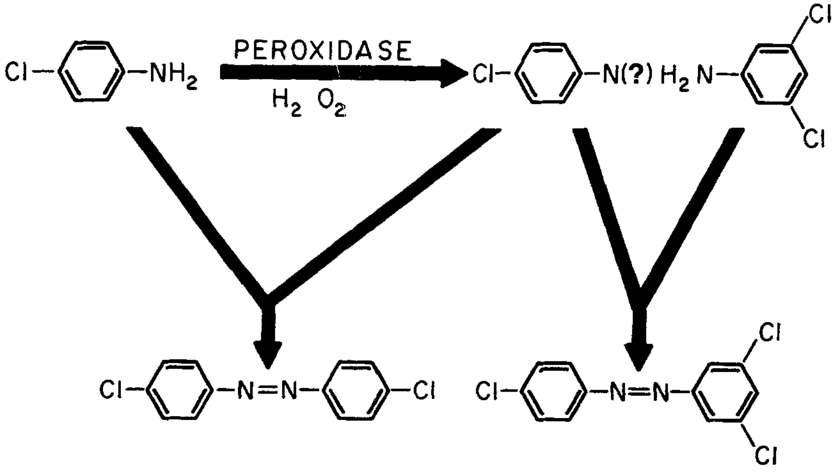

Figure 2. A labile intermediate,formed from 4-chloroaniline by peroxidase, reacts with unchanged 3,5-dichloroaniline to form 4,3',5'- trichloroazobenzene.

Isolation and identification of low amounts of reactive intermediates from complex biological systems like soil or the peroxidase reaction mixture are seldom feasible but use of labeled materials is frequently able to bypass this problem. As radiolabeled anilines were not readily available, anilines and suspected intermediates were "tagged" by appropriate ring substitutions. In the gas chromatographic system employed, the retention times of the chloroazobenzenes were directly proportional to the number of their chlorosubstituents, therefore, di-, tri- and tetra-chloroazobenzenes could readily be distinguished. The exchange of a chloro to a methyl substituent decreased the retention time of the respective azobenzene in a predictable manner (Table I).

When incubated either in soil or in the peroxidase reaction mixture, 4-chloroaniline yielded 4,4'-dichloroazobenzene and 3,4-dichloroaniline yielded 3,3',4,4'-tetrachloroazobenzene. However, when the two anilines were incubated together, besides the expected di- and tetra-chloroazobenzenes, the asymmetric 4,3',4'-trichloroazobenzene was formed. Similarly, 4-chloroaniline and 4-methylaniline, when incubated together, yielded in part the asymmetric 4-chloro-4'-methylazobenzene (Figure 1). The respective symmetric azobenzenes were also formed.

Since all these anilines were subject to transformation, these experiments did not reveal whether the labile intermediates reacted with each other, or with unchanged excess aniline. 3,5-Dichloroaniline when incubated alone in the peroxidase reaction mixture, remained unchanged (6) and was recovered quantitatively at the end of the experiment. However, if 3,5-dichloroaniline was incubated in the presence of 4-chloroaniline, in addition to 4,4'-dichloroazobenzene 4,3',5'-trichloroazobenzene was also formed (Figure 2). 3,3',5,5'-Tetrachloroazobenzene could not be detected.

This experiment clearly proved that the labile intermediate biochemically formed from 4-chloroaniline combined with unchanged 3,5-dichloroaniline to form 4,3',5'-trichloroazobenzene. Thus, the possible intermediates had been narrowed down to those that could be derived from chloroanilines by oxidative reactions and were able to combine with unchanged excess aniline to form azobenzenes. Only two compounds were considered to satisfy these requirements, phenylhydroxylamine and nitrosobenzene. 4-Chloro analogues of these compounds were incubated in soil and in the peroxidase reaction mixture both in the presence and absence of excess 4-chloroaniline. Controls with sterilized soil and inactivated peroxidase were included. These experiments showed that 4-chlorophenylhydroxylamine combined with unchanged anilines to form chloroazobenzenes. 4-Chloronitrosobenzene, when applied to soil alone, was converted to 4,4'-dichloroazoxybenzene. When applied in combination with anilines, besides 4,4'-dichloroazoxybenzene as the dominant product, small amounts of 4,4'-dichloroazobenzene were also produced. Azoxybenzenes were not normally found to be a product of aniline transformations in soil. However, it was observed that azoxybenzenes added to soil were slowly converted to their azobenzene-analogues. Phenylhydroxylamines and nitrosobenzenes are known to be in chemical equilibrium with each other (11), but the main intermediate in azobenzene formation is clearly the former. The results are consistent with the suggestion, that 4-chloroaniline is oxidized biochemically to 4-chlorophenylhydroxylamine, and the latter combines with excess 4-chloroaniline to form directly or indirectly 4,4'-dichloroazobenzene. The reactions after the formation of 4-chlorophenylhydroxylamine proceeded also in biochemically inactive media, and are, therefore, considered to be simple chemical reactions.

Summary

Formation of asymmetric azobenzenes from variously substituted aniline pairs in soil and by peroxidase pointed to the existence of labile intermediates. These were tentatively identified as the respective phenylhydroxylamines.

References

1. BARTHA, R. and PRAMER, D. Science 156, 1617 (1967).
2. BARTHA, R. J. Agr. Food Chem. 16, 602 (1968).
3. BARTHA, R., LINKE, H.A.B. and PRAMER, D. Umsch. Wiss Techn. 69, 182 (1969).
4. BARTHA, R. and PRAMER, D. Bull. Env. Cont. Toxicol., in press (1969).
5. BARTHA, R. Weed Sci. 17, in press (1969).
6. BARTHA, R., LINKE, H.A.B. and PRAMER, D. Science 161, 582 (1968).
7. LINKE, H.A.B., BARTHA, R. and PRAMER, D. Z. Naturforsch., in press (1969).
8. BRAY, H.G., JAMES, S.P. and THORPE, W.V. Biochem. J. 65, 483 (1957).
9. VOGEL, A.I. Textbook of Practical Organic Chemistry, p 630 (1948), Longmans Green, London.
10. FIESER, L.F. and FIESER, M. Advanced Organic Chemistry, p 689 (1961), Reinhold Publ. Co., London.
11. BÖTTCHER, G. and KIESE, M. Naturwissenschaften 47, 157 (1960).

Acknowledgment

This paper of the Journal Series, New Jersey Agricultural Experiment Station, was supported in part by USPHS Research Grant ES-16.