Penetration of Diphenylacetic Acid through Enzymatically-Isolated Tomato Fruit Cuticle as Influenced by Substitution on the Carboxyl Group

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Substitution on the carboxyl group of diphenylacetic acid resulted in the following decreasing order of penetration through enzymatically-isolated astomatous tomato fruit cuticle: diphenylacetic acid; *N,N*-dimethyl-2,2-diphenylacetamide; *N*-methyl-2,2-diphenylacetamide; and diphenylacetamide, respectively. Penetration was increased only in the case of the parent acid at a pH below the p*K* (3.94). pH (3 to 5) was without effect on the substituted derivatives, suggesting that permeability characteristics of the cuticle were not altered over this hydrogen ion concentration range. No strict correlation was observed between partitioning into chloroform or oleic acid and penetration. Nonionic surfactants (0.1%) increased penetration of all compounds without altering the overall penetration pattern. The marked increase in penetration of all four compounds observed after dewaxing indicated that waxes were a significant barrier, the influence being greatest in the case of the parent acid.

Although many studies relate the action of pesticides and other biologically-active compounds to their molecular structure (Albert, 1968; Blackman *et al.*, 1951; Veldstra, 1953; Woodcock, 1959), few have concentrated directly on the relationship of molecular structure to penetration (Darlington and Cirulis, 1963; van Overbeek, 1956b). Since biological activity in most plant systems is dependent on cuticular penetration, changes in biological activity based on modification of molecular structure may reflect, in part, differences in penetration (van Overbeek, 1956b).

Crafts (1961) has shown that herbicides with dissociable carboxyl groups are more effective when applied at low rather than high pH. Further, penetration of weak organic acids through the cuticle was greater in the undissociated than dissociated form (Bukovac, 1970). Lipophilic substituents have been reported (Darlington and Cirulis, 1963) to increase markedly cuticular penetration of the parent molecule; however, there may not be a strict relationship between chemical structure and partitioning into an organic solvent.

There is an increasing amount of evidence showing that molecular structure of the penetrant may have a pronounced effect on penetration through the cuticle *per se* (Darlington and Cirulis, 1963; van Overbeek, 1956b). To what extent reported biological activity–molecular structure data reflect differential penetration rather than activity at the reaction site is not known for most compounds. It is clear that a better understanding of the effect of molecular structure on penetration through the cuticle, generally regarded as the first barrier to entry of chemicals into plants (Franke, 1967; Orgell, 1957; van Overbeek, 1956a; Hull, 1970), is needed. The effect on cuticular penetration of substitution on the carboxyl group of diphenylacetic acid is the subject of this paper.

MATERIALS AND METHODS

Isolation of Cuticular Membranes. The cuticle was isolated from mature greenhouse-grown tomato fruit (*Lycopersicon esculentum* L. cv. Mich-Ohio Hybrid) using the enzymatic procedure originally developed by Orgell (1955) and modified by Norris and Bukovac (1968). The cuticle isolated in this manner was readily removed, astomatous, and experienced less damage to structural (Norris and Bukovac, 1968) or permeability properties (Norris and Bukovac, 1969) than when isolated by either mechanical or chemical isolation techniques.

Wax Removal. Dewaxed cuticles were prepared by removing both the epicuticular and cuticular waxes by Soxhlet extraction with chloroform for 2 hr (Chambers and Possingham, 1963). The extracted cuticles were then rinsed with distilled water, blotted, and dried at ambient temperature.

Synthesis of ^{14}C-Labeled Diphenylacetic Acid Derivatives. Diphenylacetic acid (DPA), diphenylacetamide (DPAm), *N*-methyl-2,2-diphenylacetamide (MDPAm), and *N,N*-dimethyl-2,2-diphenylacetamide (DMDPAm) were synthesized with ^{14}C in the carboxyl-carbon position, as described by Lemin (1966). The corresponding specific activities were 0.775, 0.600, 0.036, and 0.803 mCi/mmol.

Measurement of Penetration. The apparatus used was essentially that developed by Yamada *et al.* (1964) and modified by Norris and Bukovac (1968), except that the donor tube was suspended by a polyurethane cork containing a hole for sampling the receiver solution. One milliliter of the designated solution (1.05×10^{-5} *M*) was placed in the donor tube, while the receiver contained 25 ml of deionized water. The liquid levels were equated to eliminate hydrostatic pressure. No volume change was observed within the experimental period. Replication was three-fold. One-half or 1 ml of receiver solution was removed at designated times and transferred to 15 ml of dioxane-based scintillation fluid (Bray, 1960) and radioassayed using a Packard Tricarb scintillation spectrometer. Internal standards were used to determine efficiency.

Time-Course, pH, and Surfactant Studies. In all experiments, the concentration of the test solution was 1.05×10^{-5} *M*. For the time-course experiment, solutions were buffered with disodium hydrogen phosphate and citric acid at pH 4.0. Tergitol 15-S-9 (polyethylene glycol ether of linear alcohol) was included to provide a final concentration of 0.3%. The amount which penetrated was determined after 3, 6, 12, and 24 hr. Test solutions were buffered at pH 3.0, 4.0, and 5.0 with phosphate–citrate buffer for the pH studies. Penetration was determined after 24 hr. The nonionic surfactants Tergitol 15-S-9, Tween 20 (polyoxyethylene sorbitan

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monolaurate), and DF-16 (terminated ethoxylate of linear primary alcohols) were evaluated at 0.1% to determine their effect on penetration of DMDPAm. Penetration was determined after 24 hr. The effect of the nonionic surfactant, Tergitol 15-S-9, on penetration of the various derivatives was next established. The surfactant concentration was 0.3%, the pH was 4.0, and the penetration time was 24 hr.

Nature of the Penetrating Molecule. Thin-layer chromatography was used to confirm that the penetrant was not altered during transfer across the cuticle. After penetration, the receiver solution was acidified, extracted with chloroform, concentrated, and chromatographed. Silica gel GF was used as the solid phase and benzene:chloroform:acetic acid (85:10:5 v/v/v) was used as the developing solvent.

Relation of Molecular Structure to Partitioning. Equal portions (1 ml) of buffered test solution and chloroform or oleic acid were placed in small volumetric flasks and agitated vigorously. Each combination was replicated 5 to 6 times. After 24 hr, 0.5-ml samples were removed from both the organic and aqueous phases for radioassay.

RESULTS

Time-Course. Penetration of each compound increased with time, becoming linear after an initial lag phase (Figure 1). Initially, the quantity of the four compounds which penetrated was similar but differences became apparent with time. The following descending order of penetration after 24 hr was the parent acid (DPA), *N,N*-dimethyl-2,2-diphenylacetamide (DMDPAm), *N*-methyl-2,2-diphenylacetamide (MDPAm), and diphenylacetamide (DPAm), respectively. There was no evidence of degradation of the penetrating compounds during the course of penetration.

Effect of Donor pH. The data in Table I show that there was a significant effect of pH on penetration of the parent acid (DPA) through the cuticle. Greater penetration occurred at a pH below (3.0) rather than above (5.0) the p*K* value (3.94) of the parent acid. No pH effect was observed for DPAm, MDPAm, or DMDPAm over the pH range of 3 to 5. The absence of a pH effect with the DPA derivatives suggests that no change in the permeability of the cuticle occurs over this range of hydrogen ion concentration.

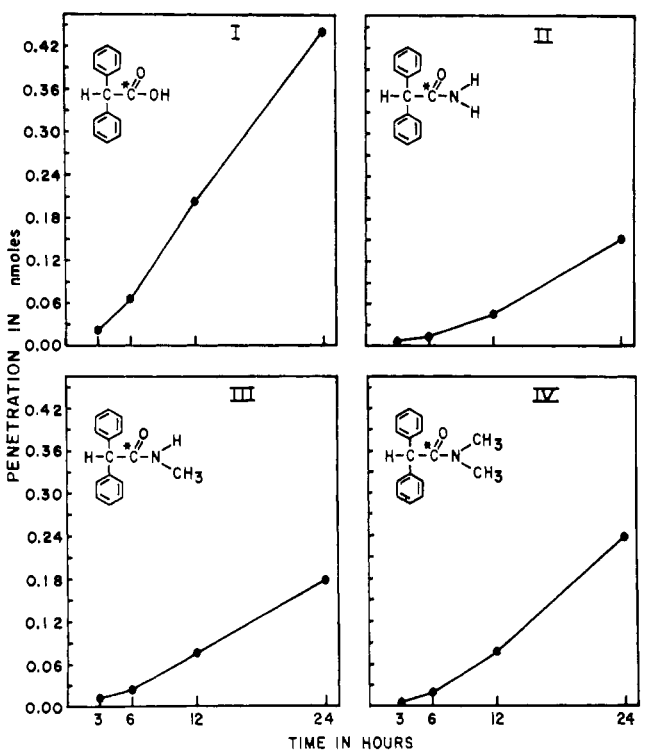

Figure 1. Time-course of penetration of diphenylacetic acid (I), diphenylacetamide (II), *N*-methyl-2,2-diphenylacetamide (III), and *N,N*-dimethyl-2,2-diphenylacetamide (IV) through enzymatically-isolated tomato fruit cuticle

Table I. Effect of Donor pH on Penetration

Compound	pH[a] 3.0	4.0	5.0
	(nmol penetrated)		
DPA	0.41a	0.37a	0.30b
DPAm	0.10a	0.13a	0.12a
MDPAm	0.18a	0.18a	0.15a
DMDPAm	0.17a	0.17a	0.17a

[a] Means within a row followed by different letters are significantly different at P = 0.01.

Table II. Effect of Surfactants (0.1%) on Penetration of DMDPAm

Surfactant	nmol Penetrated[a]	% Increase
None (control)	0.05a	...
Tergitol 15-S-9	0.13c	160
DF-16	0.11b	120
Tween 20	0.07a	40

[a] Means followed by different letters are significantly different at P = 0.01.

Table III. Effect of Tergitol 15-S-9 (0.3%) on Penetration of DPA and Derivatives

Compound	nmol Penetrated Without	Plus[a]	% Increase
DPA	0.18	0.40	122
DPAm	0.05	0.13	160
MDPAm	0.09	0.22	144
DMDPAm	0.11	0.21	91

[a] Surfactant significantly (P = 0.01) increased penetration of all compounds.

Table IV. Partition of DPA and Derivatives from Water at pH 4.0 and 7.0 into Chloroform or Oleic Acid

Compound	% Recovered in Organic Phase: Chloroform pH 4.0	Chloroform pH 7.0	Oleic Acid pH 4.0	Oleic Acid pH 7.0
DPA	94.5	66.2[a]	92.9	97.8
DPAm	95.2	96.9	94.8	97.8
MDPAm	95.0	97.6	94.8	95.8
DMDPAm	99.2	99.0	93.5	98.5

[a] Significantly different (P = 0.05) from other derivatives.

Effect of Surfactants. Penetration of DMDPAm was increased by all surfactants (0.1%) studied (Table II). Tergitol 15-S-9 was most effective, DF-16 intermediate, and Tween 20 least effective. Varying the concentration of Tergitol between 0.1 and 0.4% promoted DMDPAm penetration in each case above the control (data not reported.)

Tergitol at 0.3% promoted the penetration of all four compounds without altering the overall penetration pattern (Table III).

Relation of Molecular Structure to Partitioning. There was no significant difference (P = 0.05) in the quantity of the four compounds which partitioned into chloroform or oleic acid, with the exception of the value for DPA partitioning into chloroform at pH 7 (Table IV).

Table V. Penetration Through Dewaxed Cuticle

Compound	nmol Penetrated[a] Nondewaxed	Dewaxed[b]	% Increase
DPA	0.14	0.72	414
DPAm	0.05	0.15	200
MDPAm	0.08	0.16	100
DMDPAm	0.10	0.26	160

[a] Penetration was determined after 24 hr at pH 4.0. [b] Penetration was significantly ($P = 0.01$) greater through dewaxed than nondewaxed cuticle.

Penetration through Dewaxed Cuticle. Waxes (epicuticular and cuticular) were a barrier to penetration of all four compounds (Table V) since penetration values were greater for all four compounds through dewaxed rather than nondewaxed cuticles. The increase in penetration after dewaxing varied between compounds, DPA showing the greatest and MDPAm the least difference.

DISCUSSION

These data demonstrate that molecular structure of the penetrating molecule may play a significant role in the penetration of a foliar-applied compound through the plant cuticle. By isolating the cuticle from the underlying tissue it was possible to avoid confounding effects of the living cells and to view these data as being representative of cuticular penetration *per se*. The tomato fruit cuticle offers an excellent test system since it is astomatous and is similar both chemically and morphologically to the cuticle of leaves and fruits of a number of economic plants, frequently subjected to pesticide treatment.

Waxes acted as a barrier to penetration, as was evident by increased penetration after dewaxing. Wax removal increased penetration of all four compounds but most dramatically that of the most polar compound, namely, the parent acid (Table V). Calculations, within limits of the solvent extraction technique, revealed that epicuticular and cuticular waxes comprise 6% (or about 87 $\mu g\ cm^{-2}$) of cuticle dry weight. These waxes, as found by Brieskorn and Reinartz (1967a), are rich in triterpene alcohols and hydrocarbons in the range of C_{29} to C_{34} and such constitutents are effective barriers to penetration of polar materials (Baker and Bukovac, 1971). The cutin was found to be composed of hydroxy fatty acids with 10,16-dihydroxyhexadecanoic acid predominating (Brieskorn and Reinartz, 1967b). Shishiyama *et al.* (1970), however, found dihydroxyeicosanoic acid to be the dominant acid in tomato fruit cutin and tentatively identified eight additional hydroxy fatty acids. Dewaxing with chloroform probably results in the removal of the more nonpolar constitutents and perhaps permits greater interaction of DPA than the amides with the cutin matrix, leading to greater penetration. The enhanced penetration of all four derivatives as a result of dewaxing cannot be explained by improved wettability since dewaxing did not significantly enhance wetting. The contact angle formed by distilled water before and after dewaxing was 69.4° ± 11.7 and 73.9° ± 7.5, respectively. These values are in the intermediate region of wettability and indicate that even after wax removal the cuticle is still highly lipophilic (Holloway, 1969).

The most effective surfactant used, Tergitol 15-S-9, enhanced penetration of all four compounds to a similar degree. Thus, there appears to be no interaction of the surfactant with any of the four compounds. It is more likely that the surfactant effect is one of improving contact of the chemical with the cuticular surface through improved wetting and/or solubilization of cuticular components as envisaged by Furmidge (1959).

The descending order of penetration through enzymatically-isolated cuticle was diphenylacetic acid (DPA), *N,N*-dimethyl-2,2-diphenylacetamide (DMDPAm), *N*-methyl-2,2-diphenylacetamide (MDPAm), and diphenylacetamide (DPAm). Although lipid solubility is generally associated with preferred cuticular penetration, there was no clear difference in partition among the four compounds into chloroform or oleic acid. This was probably due, in part, to the similar water solubilities (0.133, 0.110, 0.092, and 0.250 mg/ml^{-1} at 24° C) of the four compounds. Note that the acid, least soluble in either organic phase, penetrated most.

The corresponding molecular weights are 212.24, 239.31, 225.28, and 211.25, respectively, so the acid is an exception in the sequence. Franke (1967), Orgell (1957) and Mitchell *et al.* (1960) have all stressed the importance of molecular size. The latter authors cite steric, polar, electrical (ion charge), chemical, mechanical (size to pore size), and physicochemical (competition for absorption sites) factors as the major properties of compounds influencing their interaction with the cuticle. One explanation for the greater penetration of DPA rather than the amide or methyl derivatives may be related to ease of desorption from the cuticle. Further, it must be recognized that the relative penetration of the acid in reference to the derivatives is dependent upon the pH of the donor solution, as is discussed later.

Darlington and Cirulis (1963) correlated the efficiency of penetration of a homologous series of substituted *N*-alkyl-α-chloroacetamides through isolated apricot (*Prunus armeniaca* L.) leaf cuticles. They found that although penetration was increased by lipophilic substituents, there were secondary effects which did not correlate with partition coefficients. van Overbeek (1956a) pointed out that there is a significant difference between a molecule dissolving in an oil solution and solubilizing into a membrane. In an oil solution the molecules are randomly arranged, whereas they are held in complex association in a plant membrane.

The greater penetration recorded at pH values below rather than above the pK_a value of the parent acid is in marked contrast to the lack of change in the three substituted amide derivatives. This confirms earlier data for weak organic acids (Crafts, 1957; Sargent and Blackman, 1962; Simon and Beevers, 1952) and suggests that the more lipid soluble undissociated molecules penetrate the cuticle more readily. These results parallel those of Greene (1969) on penetration of naphthaleneacetic acid (NAA) and naphthaleneacetamide (NAAm) into pear (*Pyrus communis* L.) leaves, and those of Bukovac and Norris (1966) on the effect of pH on binding of NAA and NAAm by pear leaf cuticle. All of these data provide strong evidence that the permeability of the cuticle is not altered over this hydrogen ion concentration range and that the effect of pH is primarily on the penetrating molecule rather than on the cuticle.

ACKNOWLEDGMENT

The authors thank A. J. Lemin, Upjohn Co., for the gift of radiolabeled compounds used.

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Received for review December 9, 1970. Accepted February 17, 1971. Michigan Agricultural Experiment Station Journal Article No. 5301. These studies were supported in part by Public Health Service Grant C-00246 from the National Communicable Disease Center, Atlanta, Ga., and Food and Drug Administration Grant FD-00223.

Correction

FATTY ACIDS IN TISSUE LIPIDS OF RATS FED *STERCULIA FOETIDA* OIL

In this article by E. C. Coleman and Leonard Friedman [J. AGR. FOOD CHEM. **19**(2), 224 (1971)], on page 226, under the heading **Fatty Acids,** the third sentence should read: Wood and Reiser (1965) and later Chung (1966) showed that in cyclopropanoid fatty acid metabolism the C_{19} fatty acid was converted by the β-oxidation system to a C_{13} cyclopropanoid fatty acid.