PESTICIDE EFFECTS ON THE PLANT CUTICLE

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This is to certify that the

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Martin J. Bukovac
Major professor

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ABSTRACT

PESTICIDE EFFECTS ON THE PLANT CUTICLE

By

James Albert Flore

All plant parts exposed to the environment are covered with a cuticle. The cuticle protects the plant against the external environment and is the main barrier which a foliar applied compound must transverse before penetration and a biological response can occur. Several pesticides can markedly alter cuticle development on expanding leaves. Plants with altered cuticles are often more sensitive to their environment, and to subsequently applied herbicides. A test system was developed to characterize the effect of pesticide chemicals on the development of the cuticle and to relate these findings to cuticular permeability. S-Ethyl dipropylthiocarbamate (EPTC, 2.24 kg/ha) inhibited epicuticular wax production on developing leaves of cabbage (Brassica oleracea L. var capitata cv. Market Prize). Inhibition of wax deposition was similar for abaxial (66.5%) and adaxial (67.5%) leaf surfaces. Wax bloom was visually absent from EPTC-treated plants, and its absence was associated with a marked reduction of surface fine-structure. No significant changes in cuticle thickness, structure, or

morphology were observed due to EPTC-treatment as indexed by staining with Sudan III and IV and when viewing with plane-polarized light. EPTC did not significantly affect total weight of the cuticular membrane, cuticular wax, or cutin, but there was a significant increase in the carbonate plus water soluble fraction (+33.4 $\mu g/cm^2$) which was approximately equal to the decrease in epicuticular wax weight (-28.8 $\mu g/cm^2$). EPTC altered wax composition but did not affect composition of the cutin. The alkane, ketone, and secondary-alcohol fractions of the epicuticular wax were reduced and ester content increased. C_{29} constituents (alkanes, ketones, secondary-alcohols) accounted for 71.6% (33.7 $\mu g/cm^2$) and 39.6% (7.1 $\mu g/cm^2$) of the epicuticular wax on control and EPTC-treated leaves, respectively. Homolog composition within a chemical group was not changed. Chemical composition was similar for both surfaces. In contrast with epicuticular wax, cuticular wax contained higher percentages of fatty acids and primary alcohols, and reduced alkanes and ketones. All constituents except unidentified polar compounds and fatty acids were reduced in cuticular wax extracted from EPTC-treated plants. The main component of the cutin fraction from both control and EPTC-treated plants was identified as dihydroxyhexadecanoic acid. EPTC inhibited epicuticular wax production on developing leaves of cabbage resulting in an increase in cuticular permeability as demonstrated by greater uptake of foliar applied NAA (1-naphthaleneacetic acid), Carbaryl

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(1-naphthyl N-methylcarbamate), Diphenamid (N,N-dimethyl-2,2-diphenylacetamide), $CaCl_2$ (calcium chloride), Paraquat (1,1-dimethyl-4,4-bipyridinium ion), 2,4-D (2,4-dichlorophenoxy acetic acid), but not Dieldrin (1,2,3,4,10,10-Hexachloro-exo-6,7,epoxy-1,4,4a,5,6,7,8,8a-actahydro-1,4-endo-exo-5,8-dimetholene). There was an inverse relationship between the relative partition value (partitioned between water and chloroform) and penetration. EPTC-enhanced penetration was a consequence of increased diffusion across the cuticle, and not an effect on the uptake process. Penetration of NAA increased in bean (Phaseolus vulgaris L.) and sugar beet (Beta vulgaris L.) following EPTC-treatment, and in normal and non-glossy cabbage following EPTC or trichloroacetic acid (TCA) application. The magnitude of increased penetration of NAA into leaves sampled 7 (141%) and 42 days (112%) after application was similar. Uptake in EPTC-treated and non-treated plants declined until full leaf expansion was attained (28 days after application) which coincided with maximum surface wax deposition. Uptake of silver nitrate was greater in leaves from EPTC-treated plants than non-treated plants and was preferentially taken up by the cuticular ledges of guard cells, followed by the guard cells, and the anticlinal walls of epidermal cells. Wettability and retention were significantly increased following EPTC-application. In experiments where wettability and retention were not factors, cuticular

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permeability was increased resulting from reduced epicuticular wax levels. There was an inverse relationship between wax level and cuticular transpiration.

PESTICIDE EFFECTS ON THE PLANT CUTICLE

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Guidance Committee:

The Paper-Format was adopted for this thesis in accordance with departmental and university regulations. The thesis body was separated into three sections. Each section is intended for publication in The Journal of the American Society for Horticultural Science.

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INTRODUCTION

Certain pesticides may inhibit or alter cuticle development on expanding leaves. Plants treated with these chemicals are often more sensitive to their environment, and are more susceptible to subsequently applied herbicides.

Any plant surface exposed to the external environment is covered by a thin continuous noncellular lipodial membrane, the plant cuticle. The cuticle aids in the conservation of water; prevents loss of plant components by leaching; protects the plant from damage by wind or abrasion; protects against attacks by insects or pathogens; and is the first barrier a foliar applied chemical encounters before retention and penetration occurs.

The structure and composition of the surface wax influences water repellency, which affects spray retention and the initial pesticide dose available to the plant. To induce a biological response the chemical must transverse the cuticle and be transferred to an active site. Waxes associated with the cuticle impede penetration of foliar applied chemicals into leaves, but little is known concerning the effect other cuticular components have on cuticular permeability. Therefore, any modification of cuticular structure or composition, by physical, chemical, or

environmental factors may have a profound influence on cuticular permeability and hence the cuticle's efficiency as a protective covering.

Greater plant sensitivity to pesticide chemicals or environmental stress has been associated with chemicals that alter cuticle development. These responses have been attributed to reduced wax levels, which may affect wettability, retention, or cuticular permeability. How these pesticides affect total cuticle development and influence penetration of foliar applied chemicals has not been resolved.

Accordingly, we have initiated a study designed to characterize the effect of pesticide chemicals, principally EPTC, on the development of the cuticle, and to relate these findings to cuticular permeability. We defined a test system, investigating the effect of EPTC on cuticle development in cabbage, determined the dynamics of plant response, and the conditions needed to produce a desired response to provide a basis for further studies. Using this system we then describe cuticular changes in morphology, structure, and composition induced by EPTC, and related these findings to cuticular permeability.

SECTION I

PESTICIDE EFFECTS ON THE PLANT CUTICLE: II. EPTC EFFECTS ON THE MORPHOLOGY AND COMPOSITION OF *BRASSICA OLERACEA* L. LEAF CUTICLE

Abstract. S-Ethyl dipropylthiocarbamate (EPTC, 2.24 kg/ha) inhibited epicuticular wax production by developing leaves of cabbage (Brassica oleracea L. var capitata cv. Market Prize). Inhibition of wax deposition was similar for the abaxial (66.5%) and adaxial (67.5%) leaf surfaces. Wax bloom was absent from EPTC-treated plants, and its absence was associated with a marked reduction of surface fine-structure. No significant changes in cuticle thickness, structure, or morphology were observed due to EPTC-treatment as indexed by staining with Sudan III and IV and viewing with plane-polarized light. EPTC did not significantly affect total weight of the cuticular membrane, cuticular wax, or cutin, but there was a significant increase in the carbonate plus water soluble fraction (+33.4 $\mu g/cm^2$) which was approximately equal to the decrease in epicuticular wax weight (-28.8 $\mu g/cm^2$).

Efficient pesticide performance is often dependent upon the physical, chemical and structural characteristics of the plant surface and its external covering, the plant cuticle.

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The cuticle is the first barrier that a foliar spray encounters before retention and penetration of pesticide chemicals can occur. The cuticular membrane is composed of a cutin matrix in which cuticular wax is embedded and covered on the outer surface with epicuticular (surface) wax. The cuticular membrane is separated from the cell wall by a continuous layer of pectinaceous material (19, 22,23). The structure and composition of the epicuticular wax influences wettability, retention, and cuticular permeability (3,15,20), but the contribution of the other cuticular components on these processes remains largely unknown.

Since Dewey's (7) original observation that trichloroacetic acid (TCA) inhibited epicuticular wax production in developing pea leaves, resulting in greater plant sensitivity to the external environment, similar activity has been reported for other compounds (4,8,9,10,15,16,26,27,28, 29). Recently investigators have demonstrated that certain thiocarbamates alter the quantity, chemistry, and structure of epicuticular wax (4,9,10,15,26,27,28,29), without affecting internal lipids or fatty acid content (12,17,25). However, no data are available on the effect of thiocarbamates on the cuticular wax or cutin components.

Because the cuticle is the primary protective covering, any pesticide-induced change in the composition or structure of the different cuticular components may have a profound influence on cuticular permeability. Accordingly, we have

studied the influence of EPTC on cuticle development (9), and now report the effects of EPTC on cuticle morphology and composition, so as to provide a basis for further studies on cuticular permeability.

Materials and Methods

Plant culture and EPTC application. Culture and EPTC treatment of cabbage plants was as previously described (9). Briefly, plants were treated with EPTC, 2.24 kg/ha, active chemical from a 75% emulsifiable concentrate as an aqueous soil drench (10 ml/pot), when in the 4-6 leaf stage. The youngest visible node was marked, and leaves were harvested for experimental studies from the marked node 14-21 days after herbicide application.

Scanning electron microscope observations. Fresh, freeze dried, and air dried sections from leaf samples ($5mm^2$) were attached to aluminum studs, coated with (a 200Å film of) gold/palladium alloy (Au 60%, Pd 40%), and observed with a Cambridge Stereoscan Mark 2A scanning electron microscope operated at 10kv. We observed no significant differences due to sample preparation. Scanning electron micrographs were taken on Ilford HP4 film.

Microtechnique. Leaf tissue sections were prepared similar to that described by Norris and Bukovac (18). Tissue sections ($5mm^2$) were embedded in Ames™ O.C.T. compound (Ames Co., Elkhart, Indiana) and sectioned at 12

µm with an International-Harris Cryostat, Model CTD, operating at -18 to -20°C. Sections were stained with Sudan III and IV in 95% ethyl alcohol by immersion until adequate staining occurred, rinsed and mounted in a solution of 0.2% phenol and 50% glycerol. Birefringence of non-stained sections was observed using plane-polarized light, and a first order red compensating filter. Rotation of the red compensating filter allowed for separation of birefringence according to color, here represented as light against a gray background. Photographs were taken using a Wild M20 research microscope equipped with a 35mm film carrier and a photoautomat exposure control unit. Cuticle thickness was estimated by measuring (10 observations) the thickness above the periclinal cell wall of enlarged photomicrographs taken of 5 different leaves.

Characterization of the cuticular membrane. Total epicuticular wax was determined as previously described (9). Epicuticular wax on the adaxial and abaxial surfaces was determined separately by allowing 15 ml of chloroform to flow over the surface from a fine orifice burette. Leaf cuticle (minus epicuticular wax) was isolated enzymatically. Discs ($2cm^2$) were punched from leaves, and placed in a solution of 5.0% (w/v) pectinase (Nutritional Biochem. Corp., Cleveland, Ohio) plus 0.2% (w/v) cellulase (Nutritional Biochem. Corp., Cleveland, Ohio) buffered at pH 3.7 (dibasic sodium phosphate/citric acid) and incubated at 35°C. Cuticle separation from underlying cellular material

occurred after 3-4 days. Isolated cuticles were washed in distilled water and incubated for an additional 3 days in a freshly prepared pectinase/cellulase solution. Upon removal they were washed 3 times with distilled water, and assigned to 3 groups of 200. Dry wt was determined after drying to constant wt at 40°C. Total membrane wt was determined by summation of epicuticular wax wt to the isolated membrane wt. Cuticular waxes were extracted with chloroform:methanol (1:1 v/v) under reflux for 2 hr. The extract, and cuticular membranes were dried and weighed. Carbonate soluble material was removed by refluxing the cuticular membranes with 1% sodium carbonate for 2 hr. Cutin acids were released by refluxing for 3 hr with 3% ethanolic potassium hydroxide. The hydrolysate was acidified and cutin acids were partitioned into ether. Wt of water soluble materials retained in the hydrolysate were also determined. Data are expressed on a weight/unit area basis ($\mu g/cm^2$); and are the mean of 3 determinations from each treatment.

Statistical. Data were subjected to analysis of variance and significance between treatment means was determined by the Tukey ω-procedure (24).

Results

Surface morphology. EPTC caused a marked reduction in the wax bloom of developing leaves, stems, and petioles (Fig. 1). Leaves from treated plants are almost completely

Figure 1. Photograph illustrating the bloom on control (left) and EPTC-treated (right) plants. Plants photographed 21 days after treatment.

void of epicuticular wax fine-structure on both adaxial, and abaxial leaf surfaces (Fig. 2 B, D). At 200x the stomata on control plants were obscured by surface structure, while they were clearly visible on EPTC-treated leaves (Fig. 2 A, B). The adaxial and abaxial leaf surfaces of control plants were covered by uniform tube and dendrite wax fine-structures (Fig. 2 A, C). The tubes were perpendicular to the leaf surface, and the dendrites appeared to be formed at the apex of the tubes parallel to the leaf surface (Fig. 2 A, C). Comparable structures were not observed projecting from the guard cells. Stomata, however, were obscured by structures projecting from adjacent cells (Fig. 2 A, C). The surfaces of EPTC-treated plants were lacking in tubes or dendrites, but small mounds of crystalline appearing waxes were distributed uniformly on both surfaces and appeared more dense on the adaxial than abaxial surface (Fig. 2 B, D).

Cuticle morphology. The cabbage cuticle was uniform over the leaf surface, no hairs or trichomes were observed, and there were no readily distinguishable characteristics associated with the adaxial and abaxial cuticles once isolated. The cuticular membrane was readily stained with Sudan III and IV, and was readily distinguishable from the underlying epidermal cell wall (Fig. 3 A, B). There were no significant differences in cuticle thickness between control and EPTC-treated plants, or between leaf surfaces (Fig. 3 A, B). The isolated cuticular membrane was

Figure 2. Scanning electron micrographs of surface fine-structure on cabbage leaves from control and EPTC-treated plants, 21 days after application. A: control, adaxial surface. B. EPTC, adaxial surface. C: control, abaxial surface. D: EPTC, abaxial surface. Approximate magnifications: left, 200X; center, 2000X; right, 5000X.

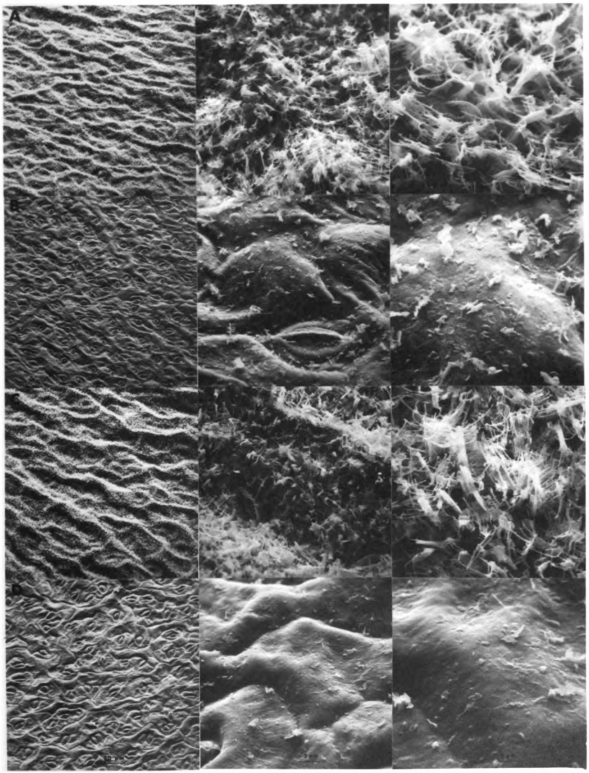
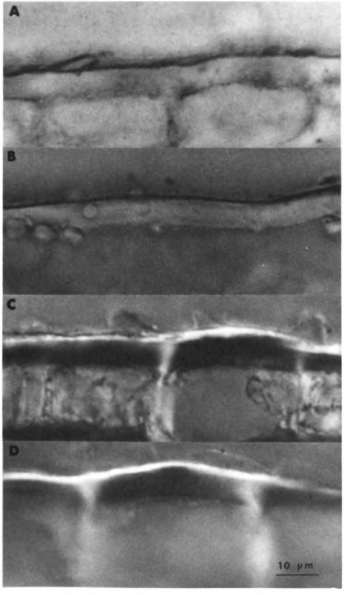


Figure 3. Cross section of cabbage leaves illustrating the cuticular membrane (stained with Sudan III and IV), and birefringence of the cuticular waxes as viewed with plane-polarized light, from control and EPTC-treated plants. Leaves were sectioned 19-21 days after EPTC application. Magnification as indicated. A: control, Sudan stain. B: EPTC, Sudan stain. C: control, plane-polarized light. D: EPTC, plane-polarized light.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extremely thin and fragile, and ranged from 0.68-0.75 µm overlying periclinal cell walls. We did not observe staining in the substomatal cavity, but because of the thinness of the cuticle it may have been ruptured during sectioning and not detected. We observed an almost continuous region of negative birefringence in both adaxial and abaxial surfaces for control and EPTC-treated plants (Fig. 3 C, D). There were no significant differences in continuity of negative birefringence between treatments, or leaf surfaces.

Characterization of the cuticular membrane. EPTC did not affect total membrane, cuticular wax, or cutin wt. There was a significant increase (33.4 $\mu g/cm^2$) in epicuticular wax (Table 1) in EPTC-treated plants. Wax accounted for 50% of the total membrane wt in control plants and only 24.5% in EPTC-treated plants. The cutin component was not significantly different between the 2 treatments. EPTC inhibited epicuticular wax deposition equally on adaxial (67.5%) and abaxial (66.5%) leaf surfaces. Within treatments, epicuticular wax deposition was not significantly different between leaf surfaces (Table 2).

Discussion

EPTC inhibited epicuticular wax production and fine-structure on developing cabbage leaves. These data are

Table 1. The effect of EPTC on the composition of cuticles isolated from developing leaves of *Brassica oleracea*.

Treatment	Cuticular component ($\mu g/cm^2$)[z]				
	Total cuticle	Epicuticular wax	Cuticular wax	Cutin	Carbonate plus water soluble
Control	108.4a	47.0a	7.0a	41.0a	13.4a
EPTC	113.1a	18.2b	9.5a	38.5a	46.8b
Change	+4.6	-28.8	+2.5	-2.5	+33.4

[z]Mean separation within a column by Tukey's ω test, P = 0.05.

Table 2. Comparison of epicuticular wax levels on the adaxial and abaxial leaf surfaces of B. oleracea as influenced by EPTC treatment.

Treatment	Epicuticular wax ($\mu g/cm^2$)[z]	
	Adaxial	Abaxial
Control	48.3a	48.1a
EPTC	15.7b	16.1b
Inhibition (%)	67.5	66.5

[z]Mean separation by Tukey's ω test, P = 0.05.

consistent with those of other researchers (4,8,9,10,15,16, 26,27,28,29). Further, EPTC had no effect on total membrane, cutin, or cuticular wax wt, but there was a significant increase in the carbonate plus water soluble fraction (Table 1).

In EPTC-treated plants an increase in the carbonate plus water soluble fraction (+33.4 $\mu g/cm^2$) almost equaled the decrease in epicuticular wax (-28.8 $\mu g/cm^2$), resulting in a nonsignificant change in total membrane wt. This increase could result from an accumulation of wax precursors due to a blockage in the wax biosynthetic pathway caused by EPTC-treatment. Kolattukudy and Brown (16) suggested that long chain surface lipids are formed by elongation and decarboxylaction of fatty acids, and proposed that thiocarbamates affect wax biosynthesis by inhibiting the elongation of fatty acids. Cellular fatty acid content is not inhibited by EPTC (12). Wilkinson and Hardcastle (29) reported that in sicklepod (Cassia obtusifolia L.) internal fatty acids increased with EPTC-application while surface hydrocarbons decreased. The increase we observed in the carbonate plus water soluble fraction is probably not due to an accumulation of fatty acids, because they are soluble in chloroform, or chloroform methanol, and would have appeared in the epicuticular or cuticular wax fractions. The fatty acids may have been diverted into other polar constituents which are not chloroform soluble, which would

require an alternate pathway, and the transfer of this material into the cuticular membrane.

Alternatively, cuticles void of wax may contain greater amounts of cellular material, which would not be completely removed by cuticular isolation, or by lipodial solvents, and therefore would be present in the carbonate plus water soluble fraction. Still et al. (26), observed that diallate increased polar compounds in pea leaves, and postulated that this was due to greater extraction of internal lipids, because of greater penetration of the lipid removing solvent into leaves having less epicuticular wax. In our experiments the cause of this increase is still not resolved. The relative importance of this fraction to foliar retention and penetration of an aqueous pesticide application is unknown. It is probably minimal because it is most likely embedded within the cutin matrix, and it has a hydrophylic character.

The influence that epicuticular and cuticular waxes have on retention and cuticular permeability is not completely understood. Epicuticular wax fine-structure and chemistry directly influence wettability (8,10,13,15) which in turn affects retention and thus the initial pesticide dose available to the plant. Removal of epicuticular or cuticular waxes by brushing, or solvent extraction, results in greater pesticide uptake (2,5,20). Norris and Bukovac (19) have suggested that cuticular wax orientation may influence cuticular permeability. In model experiments

Grncarevic and Radler (11), and Baker and Bukovac (1), demonstrated that rates of water penetration are influenced by the chemistry of the wax barrier. Norris (18) likewise suggested that differences in cuticular composition could influence permeability. Plants pretreated with pesticides known to inhibit epicuticular wax production, are often more sensitive to subsequent herbicide application (7,8,10). Therefore, epicuticular wax may influence pesticide efficiency by affecting the initial dose available for absorption, and by influencing the permeability of the wax barrier. The contribution of these factors individually to absorption remains unanswered.

We concluded that EPTC inhibits epicuticular wax production and fine-structure development, and does not quantitatively affect other cuticular components, except for an increase in the carbonate plus water soluble fraction of the cuticular membrane. Further, increased cuticular permeability is most likely due to the decreased epicuticular wax level and fine-structure as a result of EPTC application. Further investigations are in progress to ascertain the effect of EPTC on the qualitative development of the plant cuticle, and to relate changes in cuticular composition to retention, and permeability.

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Literature Cited

1. Baker, E. A., and M. J. Bukovac. 1971. Characterization of the components of plant cuticles in relation to the penetration of 2,4-D. Ann. Appl. Biol. 67: 243-253.

2. Bukovac, M. J. 1965. Some factors affecting the absorption of 3-chlorophenoxy-α-propionic acid by leaves of peach. Proc. Amer. Soc. Hort. Sci. 87: 131-138.

3. _____. 1970. Movement of materials through the plant cuticle. Proc. 18th Hort. Congr. 4:21-42.

4. Cantliffe, D. J., and G. E. Wilcox. 1972. Effect of surfactant on ion penetration through leaf wax and a wax model. J. Amer. Soc. Hort. Sci. 97:360-363.

5. Darlington, W. A., and J. Barry. 1965. Effects of chloroform and surfactants on permeability of apricot leaf cuticle. J. Agr. Food Chem. 13:76-78.

6. Davis, D. G. and K. F. Dusbabek. 1973. Effect of diallate on foliar uptake and translocation of herbicides in pea. Weed Sci. 21:16-18.

7. Dewey, O. R., P. Gregory, and R. K. Pfeiffer. 1956. Factors affecting the susceptibility of peas to selective dinitro-herbicides. Proc. 3rd. Brit. Weed Contr. Conf. 1:313-326.

8. _____, G. S. Harley, and J. W. G. MacLauchlen. 1962. External leaf waxes and their modification by root-treatment of plants with trichloroacetate. Proc. Roy. Soc. Ser. B. Biol. Sci. 155:532-550.

9. Flore, J. A., and M. J. Bukovac. 1974. Pesticide effects on the plant cuticle: I. Response of Brassica oleracea L. to EPTC as indexed by epicuticular wax production. J. Amer. Soc. Hort. Sci. 99:34-37.

10. Gentner, W. A. 1966. The influence of EPTC on external foliage wax development. Weeds. 14:27-31.

11. Grncarevic, M., and F. Radler. 1967. The effect of wax components on cuticular transpiration--model experiments. Planta. 75:23-27.

12. Harwood, J. L., and P. K. Stumpf. 1971. Fat metabolism in higher plants XLIII. Control of fatty acid synthesis in germinating seeds. Arch. Biochem. Biophys. 142:281-291.

13. Holloway, P. J. 1969. Chemistry of leaf waxes in relation to wetting. J. Sci. Fd. Agr. 20:124-128.

14. _____, and E. A. Baker. 1970. The cuticles of some angiosperm leaves and fruits. Ann. Appl. Biol. 66:145-154.

15. Juniper, B. E. 1957. The effect of pre-emergent treatment of peas with trichloroacetic acid on the submicroscopic structure of the leaf surface. New Phytol. 58:1-5.

16. Kolattukudy, P. E., and L. Brown. 1974. Inhibition of cuticular lipid biosynthesis in Pisum sativum by thiocarbamates. Plant Physiol. 53:903-906.

17. Mann, J. D., and M. Pu. 1968. Inhibition of lipid synthesis by certain herbicides. Weed Sci. 16:197-198.

18. Norris, R. F. 1974. Penetration of 2,4-D in relation to cuticle thickness. Amer. J. Bot. 61:74-79.

19. _____, and M. J. Bukovac. 1968. Structure of the pear leaf cuticle with special reference to cuticular penetration. Amer. J. Bot. 55:975-983.

20. _____, and M. J. Bukovac. 1972. Influence of cuticular waxes on penetration of pear leaf cuticle by 1-naphthaleneacetic acid. Pest. Sci. 3:705-708.

21. Pfeiffer, R. K., O. R. Dewey, and R. T. Brunskill. 1959. Further investigation of the effect of pre-emergence treatment with trichloroacetic and dichloropropionic acids on the subsequent reaction of plants to other herbicidal sprays. Proc. 4th Int. Congr. Crop Protection. 1:523-525.

22. Roelofsen, P. A. 1952. On the submicroscopic structure of cuticular cell walls. Acta. Bot. Neel. 1:99-114.

23. Sitte, P., and R. Rennier. 1963. Untersuchungen an cuticularen Zellwandschichten. Planta. 60:19-40.

24. Steel, G. D., and J. H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill Book Co., Inc., New York.

25. Stevens, V. L., J. S. Butts, and S. C. Fang. 1962. Effects of plant growth regulators and herbicides on metabolism of ^{14}C-labelled acetate in pea root tissues. Plant Physiol. 37:215-222.

26. Still, G. G., D. G. Davis, and G. L. Zander. 1970. Plant epicuticular lipids: Alteration by herbicidal carbamates. Plant Physiol. 46:307-314.

27. Wilkinson, R. E. 1974. Sicklepod surface wax response to photoperiod and S-(2,3-dichloroallyl)diisoprophythiocarbamate (Diallate). Plant Physiol. 53:269-275.

28. Wilkinson, R. E., and W. S. Hardcastle. 1969. EPTC effects on sickle pod petiolar fatty acids. Weed Sci. 17:335-337.

29. _____, and W. S. Hardcastle. 1970. EPTC effects on total fatty acids and hydrocarbons. Weed Sci. 18:125-128.

SECTION II

PESTICIDE EFFECTS ON THE PLANT CUTICLE: III. EPTC EFFECTS ON THE QUALITATIVE COMPOSITION OF *BRASSICA OLERACEA* L. LEAF CUTICLE

Abstract. S-Ethyl dipropylthiocarbamate (EPTC, 2.24 kg/ha) altered wax composition on developing leaves of cabbage (Brassica oleracea L. var. capitata cv. Market Prize), but did not affect composition of the cutin. The alkane, ketone and secondary-alcohol content of the epicuticular wax was reduced and ester content increased. C_{29} constituents (alkane, ketone, and sec-alcohol) accounted for 71.6% (33.7 µg/cm^2) and 39.6% (7.1 µg/cm^2) of the epicuticular wax on control and EPTC-treated leaves respectively. Homolog composition within a chemical group was not changed. Chemical composition was similar for abaxial and adaxial leaf surfaces, and the effect of EPTC on chemical composition was similar for both surfaces. In contrast with epicuticular wax, cuticular wax contained higher percentages of fatty acids and primary alcohols, and lower percentages of alkanes, and ketones. All constituents except the unidentified polar constituents and fatty acids were lower in cuticular wax extracted from EPTC-treated than non-treated plants. The main component of the

cutin fraction from both control and EPTC-treated plants was identified as dihydroxyhexadecanoic acid.

Several pesticide chemicals inhibit leaf cuticle development resulting in greater plant sensitivity to the external environment. Plants treated with these chemicals are often more susceptible to fungal attack or herbicide injury. They may lose water at greater rates, or retain greater quantities of an aqueous spray (3,5,10,14,17). This increased sensitivity has been associated with reduced levels of epicuticular wax. Certain thiocarbamates cause marked changes in the quantity, quality, and surface fine structure of epicuticular wax on developing leaves of pea, cabbage, and sicklepod, but little is known of their effects on the qualitative composition of other cuticular components, or how these alterations may be related to cuticular permeability (3,4,8,8,10,16,21,22,23,24).

We have initiated a study designed to establish the effects of pesticide chemicals, principally EPTC, on cuticle development and to utilize cuticles so altered in gaining a better understanding of cuticular permeability. Earlier we reported on the nature and duration of the EPTC effect on the cuticle as indexed by epicuticular wax production (8) and the effects of EPTC upon the morphology and quantitative composition of the cuticular membrane (9). We now further characterize our test system by describing the effects of EPTC on the chemical composition of cabbage leaf cuticle.

Materials and Methods

Plant culture and cuticle fractionation. Cabbage plants were treated with EPTC, 2.24 kg/ha, epicuticular waxes were isolated from developing leaves, which were in the bud stage at time of EPTC-application, cuticular membranes were isolated, and cuticular waxes and the carbonate soluble materials were extracted from the cutin matrix as previously described (9). The cutin matrix (approx. 50 mg) was refluxed for 3 hr with 25 ml 3% (w/v) sodium methanol. The reaction mixture was filtered and the residue refluxed for an additional 20 min. The combined methanolic filtrates were acidified with 2M H_2SO_4 (25 ml, 10% v/v H_2SO_4:methanol) and taken to dryness on a rotary evaporator. The residue was suspended in 50 ml distilled water and the methylated cutin acids were extracted with chloroform. This method yields esters of acids originally esterfied in the cutin polymer and prevents the formation of methoxy methyl ester artifacts (13). Using the method of Eglington et al. (7) trimethylsilyl (TMS) esters were formed from N,O-bis-(trimethylsilyl) acetamide.

Thin layer chromatography. Epicuticular and cuticular wax constituents were separated by TLC, and chemical classes were identified by comparison with standards, or published RF values (1,18,19). Waxes were dissolved in chloroform (10 mg/ml, w/v) and spotted (5µl) on precoated silica gel G thin-layer plates (Uniplate^R, 250 microns, Analtech, Inc.,

Newark, Delaware), which were prewashed in distilled benzene and dried at 110°C for 30 min. Spotted plates were developed in benzene, and wax constituents were localized by charring (160°C) after spraying with H_2SO_4, or by reacting with iodine vapor. For quantitative TLC epicuticular wax (200mg) from control and EPTC-treated plants were streaked as a narrow band (2mm) on each of 10 thin-layer plates. The plates were developed in benzene, constituents were localized by reacting with iodine vapor, and their respective areas were scraped from the plates and recovered by refluxing with chloroform for 2 hr. Chloroform was evaporated and wax quantity determined by weight. Complete separation of each fraction was confirmed by TLC. Each chemical group was further analyzed qualitatively by GLC. Qualitative separation of epicuticular waxes form abaxial and adaxial leaf surfaces, and of cuticular waxes for GLC analysis was achieved by streaking 5mg of wax on thin-layer plates, followed by development and recovery using the above procedure.

Gas liquid chromatography. All GLC data were obtained using a Packard 7300-gas liquid chromatograph equipped with a hydrogen flame ionization detector and a temperature programmer. The column was stainless steel (2mm I.D., 5.8m length) packed with Chromosorb W 80/100 mesh, coated with 1.25% SE-30. Operating conditions were: nitrogen flow 40 ml/min, inlet and detector temp 360°C, and column temperature programmed from 120 to 350°C at 6°C per min.

Fatty acids were methylated using diazomethane (20), all other chemical groups were detected without conversion to a derivative. Unknowns were identified by comparing elution times with elution times of known standards. Standard curves were constructed from: n-alkanes C-22, C-24, C-28, C-32, C-36 (Analabs); primary alcohol C-22 (J. T. Baker Co.); C-26, C-28 (Analabs); methyl esters of fatty acids C-12, C-14, C-16, C-18, C-20, C-22 (Packard Instruments); aldehydes C-18 (Analabs); C-24, C-26, C-28 (isolated from Chenodpodium album); ketone C-35 (J. T. Baker Co.); secondary alcohol C-29 (isolated from Brassica oleracea L.); esters C-32, C-36 (Analabs); C-46 (gift from A. P. Tulloch, Prairie Regional Lab., Saskatoon, Saskatchewan); C-40, C-42 (synthesized). Relative retention (T rel) data were determined by comparing unknown and unidentified elution times with elution times of internal standards: n-tetracosane for whole wax, alkanes, ketones, and secondary alcohols; 1-docosanol for primary alcohols; methyl docosanate for methyl esters of fatty acids; and octadecyl sterate for long chain esters. Quantification was determined by peak areas (height x base at 1/2 peak height). Corrections were made for detector response based on n-octacosane. All data are the means of 3 determinations per sample.

Cutin acid identification. Cutin acids were converted to TMS ether methyl esters and were separated by GLC using the same conditions as for wax analysis, except temperature

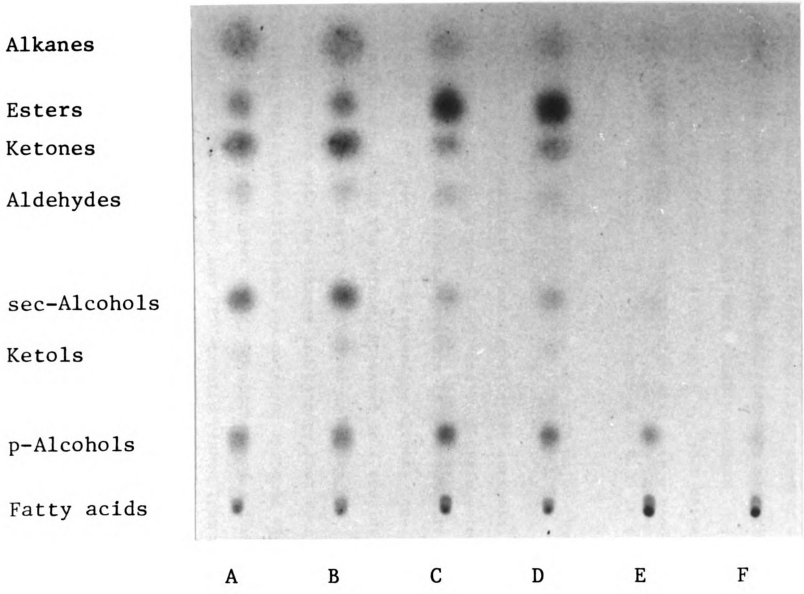
was programmed at 5°C/min 120-280°C, and inlet and detector temperature was 290°C. Unknowns were compared with TMS ether methyl esters of hexadecanoic acid, ω-hydroxypentadecanoic acid, ω-hydroxyhexadecanoic acid dihydroxyhexadecanoic acid, 9,10,16-trihydroxyoctadecanoic acid (gift from E. A. Baker, Long Ashton Research Sta., UK) and identification was confirmed by GLC separation on a 1% SE-30 column. Unidentified constituents are expressed in terms of T rel for ω-hydroxyhexadecanoic acid.

Results

Wax composition-qualitative TLC. Cabbage epicuticular and cuticular waxes were clearly resolved by TLC into major chemical classes. Comparisons of epicuticular and cuticular waxes indicate marked differences in composition due to EPTC-treatment. When compared to the control epicuticular wax esters increased, and alkanes, ketones, and secondary alcohols decreased. In contrast, all chemical classes except fatty acids of cuticular waxes were found in smaller quantities as a result of EPTC-treatment (Fig. 1). Chemical composition was similar for adaxial and abaxial leaf surfaces, and the effect of EPTC on chemical composition was similar for both surfaces, Fig. 1.

EPTC effect on epicuticular wax composition. Epicuticular wax deposition on developing leaves of EPTC-treated plants was 61.7% less than the control (control 47.0 $\mu g/cm^2$;

Figure 1. Thin-layer chromatogram of epicuticular and cuticular waxes isolated from developing leaves of control and EPTC-treated plants.
A: epicuticular, control, adaxial surface.
B: epicuticular, control, abaxial surface.
C: epicuticular, EPTC, adaxial surface.
D: epicuticular, EPTC, abaxial surface.
E: cuticular, control. F: cuticular, EPTC.


Alkanes
Esters
Ketones
Aldehydes
sec-Alcohols
Ketols
p-Alcohols
Fatty acids
A
B
C
D
E
F

EPTC 18 $\mu g/cm^2$), Table 1. All chemical constituents of the epicuticular wax were not affected equally. Alkanes (-8.8 $\mu g/cm^2$), ketones (-13.4 $\mu g/cm^2$), and secondary alcohols (-4.5 $\mu g/cm^2$) production was most notably reduced, and long chain esters were increased (+2.4 $\mu g/cm^2$). When expressed as a percentage of total epicuticular wax, esters become the most dominant constituent (34.5% compared to 8.3% in control), Table 2.

Separation of the epicuticular wax into major chemical groups was accomplished by GLC. This method provided a fast method for qualitative and quantitative analysis. The major alkane (C-29), ketone (C-29) and long chain esters are clearly resolved when temperature is programmed from 120-340°C at 6°C per min. The secondary alcohol (co-chromatographed with the ketone) appeared as a shoulder on the ketone peak. Primary alcohols and aldehydes co-chromatographed with the alkanes and ketones, but because of their small quantities and low detector response they contributed little to peak areas. Comparison of epicuticular wax from control and EPTC-treated plants from several experiments, and from abaxial and adaxial surfaces (lowered alkanes, ketones; increased esters) confirmed the TLC data, Fig. 2.

EPTC-treatment did not alter homolog composition of epicuticular wax within a chemical class. There was a marked reduction of C-29 constituents. The C-29 constituents accounted for 39.6% (7.1 $\mu g/cm^2$) of the

Table 1. Chemical composition of epicuticular wax isolated from developing leaves of control and EPTC-treated cabbage plants.

Chemical class	Treatment[z]				Change
	Control (% of total)	EPTC	Control ($\mu g/cm^2$)	EPTC	
Alkanes	26.0	18.9	12.2	3.4	-8.8
Esters	8.3	34.5	3.9	6.3	+2.4
Ketones	35.9	19.4	16.9	3.5	-13.4
Aldehydes	5.5	6.0	2.6	1.1	-1.5
secondary alcohols	10.7	3.0	5.0	0.5	-4.5
Ketols	2.2	1.8	1.0	0.3	-0.7
primary alcohols	7.6	11.6	3.6	2.1	-1.5
Fatty acids	3.9	4.8	1.8	0.8	-1.0

[z]Identification and quantity by TLC.

Table 2. Percent composition of epicuticular wax isolated from developing leaves of control and EPTC-treated cabbage plants.[z]

Chemical class	Carbon number	T. rel.	Control (%) of total	EPTC[y]
Alkanes	27		tr	1.0
	28		0.1	0.1
	29		25.0	17.5
	30		0.1	0.1
	31		0.6	0.1
Esters	40-47		8.3	34.5
Ketones	29		35.9	19.3
Aldehydes	26		1.3	1.3
	27		0.8	0.5
	28		1.4	3.0
	29		0.9	0.6
	30		1.1	0.7
secondary alcohols	29		10.7	2.8
Ketol[x]			2.2	1.8
primary alcohols[w]	24	1.19	0.5	0.9
	26	1.31	2.3	6.3
		1.39	1.7	--
	28	1.49	1.3	1.6
		1.58	1.1	1.1
		1.61	0.8	1.5
Fatty acids[u]	14	0.30	0.2	0.3
	15	0.38	--	0.2
	16	0.47	0.1	0.1
		0.67	--	0.1
	22	1.00	0.1	0.3
		1.17	0.2	0.1
		1.24	0.1	0.1
	26	1.33	1.3	0.5
		1.42	0.1	0.1
	28	1.48	1.4	1.3
		1.56	0.1	0.5
	30	1.63	0.3	1.0
		1.75	--	0.7

[z]Determined by GLC.

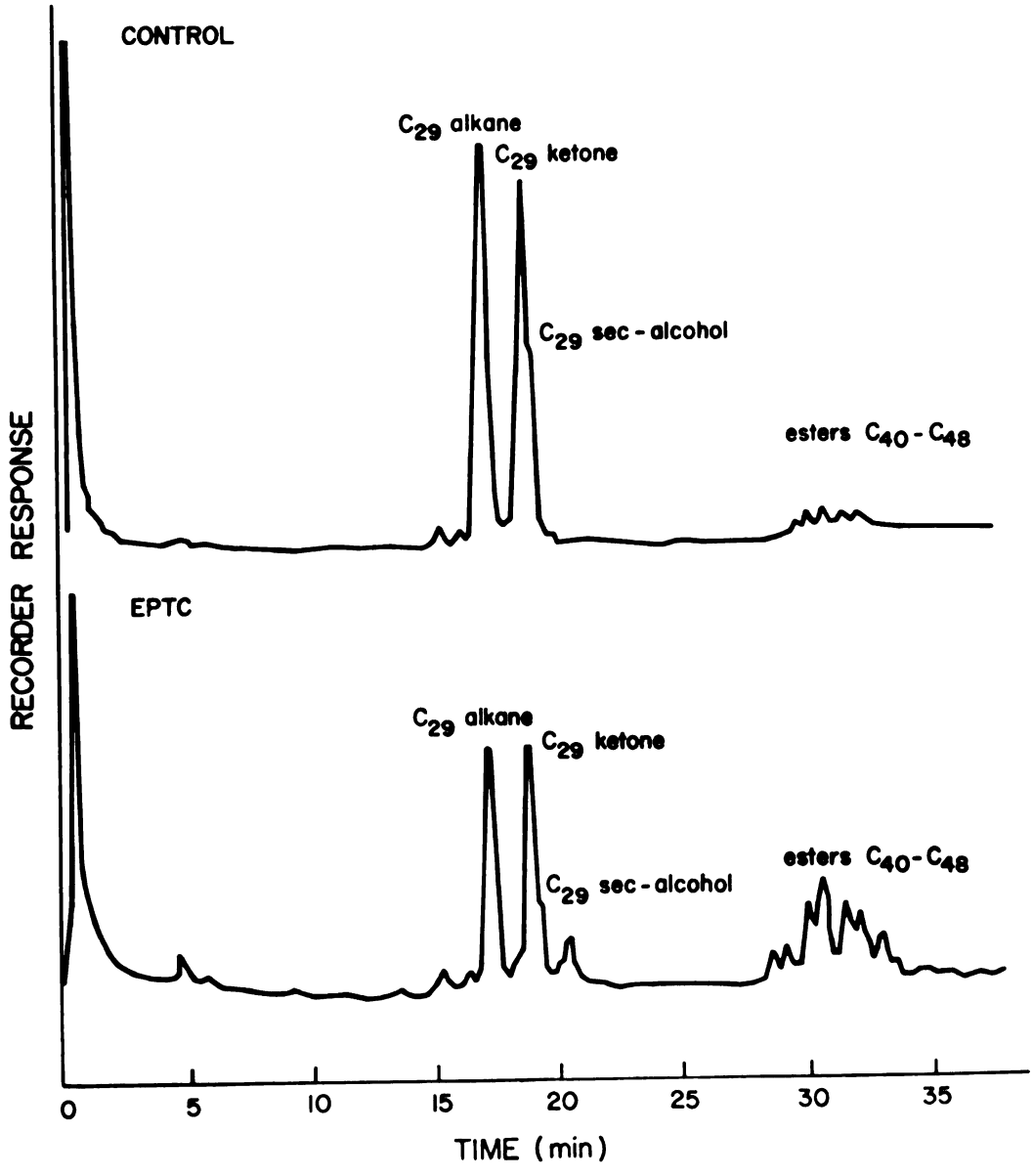
[y]Control = 47.0 μg/cm^2; EPTC-treated = 18.0 μg/cm^2.

[x]Identified by TLC rf.

[w]T rel based on 1-docosanol.

[u]T rel based on methyl docosanate.

Figure 2. GLC-traces of epicuticular wax isolated from developing leaves of control and EPTC-treated cabbage plants. (Column 1.25%, SE-30 programmed 120-350°C at 6°/min.)


CONTROL
C_{29} alkane
C_{29} ketone
C_{29} sec - alcohol
esters C_{40} - C_{48}
EPTC
C_{29} alkane
C_{29} ketone
C_{29} sec - alcohol
esters C_{40} - C_{48}
RECORDER RESPONSE
0
5
10
15
20
25
30
35
TIME (min)

epicuticular wax in EPTC-treated plants compared with 71.6% (33.7 $\mu g/cm^2$) for the controls, Table 2.

EPTC effect on cuticular wax composition. There were no significant quantitative differences in cuticular waxes between control (7.0 $\mu g/cm^2$) and EPTC-treated (9.0 $\mu g/cm^2$) plants, Table 3. Cuticular wax from EPTC-treated plants was lower in all chemical constituents, with increased amounts of fatty acids, and unidentified polar materials, when compared to cuticular wax isolated from control plants, Table 3. When expressed on a percentage basis, regardless of treatment, cuticular waxes contained more polar materials (fatty acids, primary alcohols, secondary alcohols) than epicuticular waxes, Table 2, 3.

Cutin composition. EPTC-treatment did not affect cutin composition, Table 4. The major cutin constituent (66.9% for control; 59.5% for EPTC-treated) was identified by GLC as dihydroxyhexadecanoic acid, with smaller amounts of hexadecanoic acid, octadecanoic acid and ω-hydroxy-hexadecanoic acid.

Discussion

EPTC altered the quantity and composition of epicuticular wax on developing cabbage leaves. The chemical composition of the epicuticular and cuticular waxes were modified, but EPTC did not alter the composition of the cutin. EPTC-induced alteration of cuticle development

Table 3. Chemical composition of cuticular waxes isolated from developing leaves of control and EPTC-treated cabbage.

Chemical group	Control (%) of total[y]	EPTC[x]
Alkanes	18.7	9.3
Esters	6.3	1.7
Ketones	15.8	1.6
Aldehydes	2.4	tr
secondary alcohols	6.9	tr
primary alcohols	20.5	6.7
Fatty acids	24.9	61.9
Unidentified polar compounds	4.5	19.2

[x]Control = 7.0 μg/cm^2; EPTC-treated = 9.0 μg/cm^2.

[y]Determined by GLC.

Table 4. Percent composition of cutin isolated from developing leaves of control and EPTC-treated cabbage plants.

Chemical constituent		Control (%) of total	EPTC
Hexadecanoic acid		5.8	5.1
Octadecanoic acid		1.9	1.7
ω-hydroxyhexadecanoic acid		10.1	7.4
Dihydroxyhexadecanoic acid		66.9	59.5
Unknown T rel[z]	1.04	5.0	--[y]
	0.92	10.3	9.2
	0.83	--	13.7
	0.46	--	3.4

[z]Based on ω-hydroxyhexadecanoic acid.

[y]--not detectable.

occurred primarily in the wax fractions and therefore, may affect cuticular permeability and hence the cuticle's efficiency as a protective covering.

Current concepts (15) on biosynthesis of surface wax in Brassica have been investigated utilizing isotope labeling procedures. Briefly, results indicate that long chain constituents (greater than C_{16}) are produced by the addition of acetate units to existing fatty acids until a chain length of 30 carbons is attained, followed by decarboxylation to form the major alkane, n-nonocosane. N-nonocosane is further oxidized to form the major secondary alcohol, and ketone constituents, nonocoson-14-ol, nonocoson-15-ol, and nonocoson-15-one (15). The aldehyde and primary alcohol chain lengths are similar (C_{26}, C_{28}) and of sufficient length to be derived by the reduction of fatty acids from the fatty acid elongation pathway. The combination of free primary alcohols with endogenous fatty acids to form esters is probably enzyme mediated. The free primary alcohol chain length is similar to that of the alcohol found in the wax ester which suggests a possible common origin (15). Variation in the fatty acid moiety of the ester indicates an origin from different sources (15).

Deposition of all chemical classes of the epicuticular wax fraction of the cuticle except esters, were inhibited by EPTC application (Table 1). Further, EPTC differentially inhibited the alkane, ketone, and secondary alcohol fractions, which are primarily C-29 in chain length, and

increased the long chain ester fraction (Table 2). These data indicate a decrease of nonocosane, and its oxidation products, and would support Kolattukudy's hypothesis that thiocarbamates inhibit the elongation of fatty acids which are decarboxylated to form the C-29 alkane fraction (16). Kolattukudy and Brown (16) have shown a concn dependent inhibition of wax constituents in pea by application of thiocarbamates. Synthesis of alkanes, secondary alcohols, and ketones are most sensitive, followed by primary alcohols, aldehydes, and long chain esters. Low concentrations of thiocarbamate stimulated wax ester synthesis.

The chemical composition of cuticular wax extracted from EPTC-treated cabbage was different from that of the control, however, the alteration in chemistry was not the same as observed in the epicuticular wax fraction (Table 1, 3). When expressed as a percentage of total cuticular wax, all chemical constituents except fatty acids (+37.0%) and the unidentified polar compounds (+14.7%) were reduced as a result of EPTC treatment. There was no clear differential inhibition of the alkanes, ketones, and secondary alcohols, or a stimulation of ester synthesis, as observed in the epicuticular wax fraction. Chemical classes containing long chain compounds (alkanes, ketones, esters, aldehydes, secondary alcohols, and primary alcohols) were reduced by 51.6% when compared to the control (Table 3).

Generally the distinction between epicuticular and cuticular wax is based on the location and the method

utilized to extract wax from the cuticular membrane. Often no differentiation is made between the two. Refluxing isolated cuticles with chloroform or chloroform:methanol (1:1) is usually employed to remove cuticular wax embedded within the cutin matrix (1,9). We observed that cuticular wax composition was similar to epicuticular wax, but contained higher percentages of polar compounds (fatty acids and primary alcohols) (Table 1, 3) which is similar to the observations of Baker and Bukovac (1), who investigated the epicuticular and cuticular wax chemistry of several weed species.

The differential effect of EPTC on the alteration of cuticular wax in comparison with epicuticular wax is not inconsistent with the hypothesis that fatty acid elongation is inhibited. Although, alkanes, ketones, and secondary alcohols were not discriminately reduced as in epicuticular wax, their presence was significantly lower than in the control (Table 1, 3). The marked reduction of all constituents, except the fatty acids and unidentified polar compounds in cuticular wax may have resulted from greater solvent penetration, due to the reduced amount of wax on treated plants. It could be suggested from the data that epicuticular and cuticular wax biosynthesis and deposition are affected differently by EPTC; but this is unlikely, as it would require the presence of an alternate biosynthetic pathway, or means of deposition, which are not known to exist.

When expressed as a percentage of total epicuticular wax, EPTC caused a marked change in the chemical composition (Table 1). Esters become the most dominant constituent (34.5% compared to 8.3% in the control).

Epicuticular wax chemistry may affect the wettability and retention of aqueous spray solutions (12), but little direct evidence is available concerning the influence it may have on cuticular permeability. In experiments using model systems, attempts have been made to assess the influence of wax on passage of water through artificial membranes impregnated with various epicuticular wax components (2,11). Permeability to water is dependent on the quantity, and the quality of the wax present. When plated in equal amounts esters and alcohols are more permeable than alkanes. This evidence would imply that cuticular permeability to polar compounds after EPTC alteration, may be greater because of a higher percentage of esters.

It is clear that inhibition of cuticle development results in greater subsequent injury to herbicidal sprays (5,10,17). Epicuticular wax morphology and chemistry influence retention, and cuticular permeability, unfortunately the contribution of each factor to increased efficiency in pesticide application is not completely understood. Cuticles with modified epicuticular wax chemistry may offer a unique test system to further our

understanding of wax in relation to retention and penetration of pesticide chemicals.

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Literature Cited

1. Baker, E. A. 1972. The effect of environmental factors on the development of leaf wax of Brassica oleracea var Gemnifera. MSc. Thesis, Univ. Bristol. 134 pp.

2. _____, and M. J. Bukovac. 1971. Characterization of the components of plant cuticles in relation to the penetration of 2,4-D. Ann. Appl. Biol. 67: 243-253.

3. Cantliffe, D. J., and G. E. Wilcox. 1972. Effects of surfactant on ion penetration through leaf wax and a wax model. J. Amer. Soc. Hort. Sci. 97:360-363.

4. Davis, D. G., and K. F. Dusbabek. 1973. Effect of diallate on foliar uptake and translocation of herbicides in pea. Weed Sci. 21:16-18.

5. Dewey, O. R., P. Gregory, and R. K. Pfeiffer. 1956. Factors affecting the susceptibility of peas to selective dinitro-herbicides. Proc. 3rd. Brit. Weed Contr. Conf. 1:313-326.

6. _____, G. S. Harley, and J. W. G. MacLauchlen. 1962. External leaf waxes and their modification by root-treatment of plants with trichloroacetate. Proc. Roy. Soc. Ser. B. Biol. Sci. 133:532-550.

7. Eglington, G., D. H. Hunneman, and A. McCormick. 1968. Gas chromatographic mass spectrometric studies of long chain hydroxy acids III. The mass spectra of the methyl esters trimethylsilyl ethers of aliphatic hydroxy acids. A facile method of double bond location. Org. Mass. Spectro. 1:593-611.

8. Flore, J. A., and M. J. Bukovac. 1974. Pesticide effects on the plant cuticle: I. Response of Brassica oleracea L. to EPTC as indexed by epicuticular wax production. J. Amer. Soc. Hort. Sci. 99:34-37.

9. _____. 1974. Pesticide effects on the plant cuticle: II. EPTC effects on the morphology and composition of Brassica oleracea L. leaf cuticle. J. Amer. Soc. Hort. Sci. manuscript concurrently submitted.

10. Gentner, W. A. 1966. The influence of EPTC on external foliage wax development. Weeds. 14:27-31.

11. Grncarevic, M., and F. Radler. 1967. The effect of wax components on cuticular transpiration -- model experiments. Planta. 75:23-27.

12. Holloway, P. J. 1969. Chemistry of leaf waxes in relation to wetting. J. Sci. Fd. Agr. 20:124-128.

13. _____, and A. H. B. Deas. 1971. Methoxy methyl ester artifacts in the methylation of hydroxy-fatty acids. Chemistry and Industry. p. 1140.

14. Juniper, B. E. 1959. The effect of pre-emergent treatment of peas with trichloroacetic acid on the submicroscopic structure of the leaf surface. New Phytol. 58:1-5.

15. Kolattukudy, P. E., and T. J. Walton. 1973. The biochemistry of plant cuticular lipids. Prog. Chem. Fats Other Lipids. 13:119-175.

16. _____, and L. Brown. 1974. Inhibition of cuticular lipid biosynthesis in Pisum sativum by thiocarbamates. Plant Physiol. 53:903-906.

17. Pfeiffer, F. K., O. R. Dewey, and R. T. Brunskill. 1959. Further investigation of the effect of pre-emergence treatment with trichloroacetic and dichloropropionic acids on the subsequent reaction of plants to other herbicidal sprays. Proc. 4th Int. Congr. Crop Protection. 1:523-525.

18. Purdy, J. S., and E. V. Truter. 1963. Constitution of the surface lipid from leaves of Brassica oleracea (var. capitata (Winningstadt)). I. Isolation and quantitative fractionation. Proc. Royal Soc. Ser. B. 158:536-543.

19. _____. 1963. Constitution of the surface lipid from leaves of Brassica oleracea (var. capitata (Winningstadt)). III. Nonocasane and its derivatives. Proc. Royal Soc. Ser. B. 158: 553-565.

20. Schlenk, H., and J. L. Gellerman. 1960. Esterification of fatty acids with diazomethane on a small scale. Anal. Chem. 32:1412-1414.

21. Still, G. G., D. G. Davis, and G. L. Zander. 1970. Plant epicuticular lipids: Alteration by herbicidal carbamates. Plant Physiol. 46:307-314.

22. Wilkinson, R. E., and W. S. Hardcastle. 1969. EPTC effects on sickle pod petiolar fatty acids. Weed Sci. 17:335-337.

23. _____. 1970. EPTC effects on total leaflet fatty acids and hydrocarbons. Weed Sci. 18:125-128.

24. _____. 1974. Sickle pod surface wax response to photoperiod and S-(2,3-dichloroallyl)-diisopropylthiocarbamate (Diallate). Plant Physiol. 53:269-275.

SECTION III

PESTICIDE EFFECTS ON THE PLANT CUTICLE: IV. THE EFFECT OF EPTC ON PERMEABILITY OF *BRASSICA OLERACEA* L. LEAF CUTICLE

Abstract. S-Ethyl dipropylthiocarbamate (EPTC, 2.24 kg/ha) inhibited epicuticular wax production on developing leaves of cabbage (Brassica oleracea L. var. capitata cv. Market Prize), resulting in an increase in cuticular permeability as demonstrated by greater uptake of ^{14}C-NAA (^{14}C-1-naphthaleneacetic acid) and increased cuticular transpiration. EPTC-enhanced penetration was a consequence of increased diffusion across the cuticle, and not an effect on the uptake process. Penetration of NAA was increased in bean (Phaseolus vulgaris L.) (200%), and sugar beet (Beta vulgaris L.) (121%), following EPTC application. The magnitude of increased penetration of NAA into leaves sampled at 7 days (141%) and 42 days (112%) after application was similar. Penetration in both treatments declined until full leaf expansion was attained (28 days after application). Uptake of silver nitrate was greater in leaves isolated from EPTC-treated plants than non-treated plants and was preferentially taken up by the cuticular ledges of guard cells, and the anticlinal walls of epidermal cells.

Several herbicides inhibit epicuticular wax production on developing leaves (3,6,11,12,13,18,23,24,25,26). Associated with this chemical effect is an increase in wettability, spray retention and phytotoxicity due to subsequent foliar application of herbicides (8,13,21). Epicuticular wax fine-structure (17) and chemistry (16) influences wettability, and may affect pesticide diffusion through the cuticle (2,19,20).

Recently we have demonstrated that EPTC significantly inhibited wax production, and altered the chemical composition, and surface fine-structure of epicuticular wax on developing cabbage leaves, but did not affect the composition of the cutin (12). In this paper we characterize cuticular permeability and relate these findings to changes in wax development induced by EPTC-treatment.

Materials and Methods

General. Plant culture, EPTC (2.24 kg/ha, root applied) treatment, leaf sampling and epicuticular wax determination were according to the procedures previously described (10).

Measurement of penetration. Penetration was measured utilizing a leaf disc method similar to that described by Greene and Bukovac (13). Glass vials (1.0 cm or 1.6 cm i.d.) were affixed to the leaf surface with silicone rubber (General Electric RTV-11, General Electric Co., Waterford,

N. Y.) hardened with T-11 catalyst (Wacher Co., Munich, Germany), and placed in Petri dishes lined with moistened filter paper. ^{14}C-1-naphthaleneacetic acid, 16.0 µc/mole, 6.2 X 10^{-6}M, buffered at pH 3.2 with phosphate (0.025M) citrate (0.038M) buffer was added to each vial. The following experimental conditions were standardized: temp 25 ± 1/2°C; illumination, 1.08 X 10^{4} lux, fluorescent light; penetration time period of 12 hr; treating solution, 0.025 µc; unless otherwise indicated. At the end of the penetration period the glass vials were removed, the leaf discs were thoroughly washed with distilled water, blotted dry, placed treatment side down in a 2.5 cm planchet lined with double sticky tape, and dried in an oven at 60°C for a minimum of 12 hr. Radioactivity was determined with a Beckman Low Beta II proportional gas flow counter. Corrections for background were made where applicable.

Cuticular transpiration. Leaves were detached and lanoline was applied to the cut petiole. After 1 hr, stomatal closure was assured with silicone rubber impressions. Leaves were placed in a growth chamber (dark, 25°C), and weighed at 1 hr intervals on an analytical balance. Leaf area was determined as previously described (10). Data are expressed as mg wt loss per cm^2 leaf surface per 20 hr. Since stomata were closed loss in wt was attributed to cuticular transpiration.

Species response. Cabbage (Brassica oleracea L.), sugar beet (Beta vulgaris L.), and bean (Phaseolus vulgaris

L.) were grown and treated with EPTC as previously described, with the following modifications: for bean EPTC was applied at 4.0 kg/ha 6 days after seeding and the primary leaves were sampled 7 days later; for sugar beet, EPTC was applied at 3.0 kg/ha, plants in the 2 leaf stage, and the leaves for analysis were harvested 21 days later from those nodes which were in the bud stage at time of application. Epicuticular wax levels and penetration of ^{14}C-NAA were determined as previously described with experimental conditions as indicated in Table 1.

Characterization of NAA penetration. Time course and leaf surface response was assessed by measuring penetration of ^{14}C-NAA at designated time intervals. Expansion of leaves during the penetration period limited our studies to 24 hr. The effect of NAA concn was determined by varying the treating solution concn by the addition of non-labeled NAA or dilution with buffer. Temperature response was assessed by holding petri dishes containing leaf discs in temperature regulated water baths of 5, 15, 25, and 35°C, accuracy ± 1/2°C. The effect of light was determined by comparing uptake of ^{14}C-NAA in 1.08 X 10^4 lux, fluorescent light, with that in darkness.

Response to EPTC concn. The effect of increasing concentrations of EPTC (0.00, 0.28, 0.56, 1.12, 2.24 kg/ha) on penetration of ^{14}C-NAA, cuticular transpiration, and epicuticular wax was determined according to procedures outlined above.

Table 1. Penetration of ^{14}C-NAA, and epicuticular wax deposition on developing leaves of control and EPTC-treated cabbage, bean and sugar beet plants.

Plant[z]	Penetration ^{14}C-NAA (cpm/disc)			Epicuticular wax (µg/cm^2)		
	Control	EPTC	Increase (%)	Control	EPTC	Decrease (%)
Cabbage[y]	143a	1031b	621	67.0a	34.0b	49
Bean[x]	269a	805b	200	6.2a	4.7a	24
Sugar Beet[w]	203a	448b	121	7.0a	6.2a	11

[z]Means within a row for each parameter followed by a different letter are significantly different at p = .05, Tukey ω-procedure.

[y]NAA 6.2 X 10^{-6}M, 12 hr absorption time, upper surface, 21 days after treatment.

[x]NAA 1.2 X 10^{-5}M, 7 hr absorption time, upper surface, 21 days after treatment.

[w]NAA 3.1 X 10^{-6}M, 2 hr absorption time, upper surface, 7 days after treatment.

Duration of EPTC response. Duration of the EPTC response was indexed by determining the penetration of ^{14}C-NAA by using the leaf produced at the 7th node 14, 21, 28, 35, and 42 days after EPTC application for control and EPTC-treated cabbage plants.

Localization of penetration pathways. An attempt was made to localize areas of preferential penetration, using silver nitrate, which upon reduction within the leaf can be viewed with a light microscope as discrete black metallic grains of silver. Leaf discs were prepared as for penetration studies except that the treating solution consisted of 0.1M silver nitrate containing 0.01% X-77 (alkylarylpolyoxyethylene glycols, free fatty acids, and isopropanol) in place of NAA. Penetration into the upper leaf surface was permited for 15 or 60 min, after which the vials were removed and the excess treating solution was washed off with distilled water. The leaf discs were then cleared in 95% ethanol, and localization of silver recorded by photomicrography (Wild M-20 research microscope). Leaf discs similarly treated were scanned for silver with an electron X-ray analyzer to confirm location of silver deposition.

Statistical. Where applicable data were subjected to analysis of variance and treatment means were compared by the Tukey ω-procedure (22).

Results

Species response. Significantly greater quantities of ^{14}C-NAA penetrated into cabbage (+621%), bean (+200%), and sugar beet (+121%) leaves of EPTC-treated than non-treated plants. EPTC-treatment resulted in less epicuticular wax on leaves of cabbage, but not on bean or sugar beet. Leaves from non-treated cabbage plants (67.0 µg/cm^2) had approximately 10 times more epicuticular wax than did non-treated bean (6.2 µg/cm^2) or sugar beet (7.0 µg/cm^2) leaves (Table 1).

Dynamics of uptake. Penetration of ^{14}C-NAA into leaves isolated from non-treated and EPTC-treated plants was linear with time for both surfaces. Rates of uptake were greatest for leaves from EPTC-treated plants, and the abaxial surfaces were more permeable than adaxial surfaces (Fig. 1). Penetration was linear for increasing concentrations of NAA for both non-treated and EPTC-treated plants. Penetration increased with an increase in temperature, and was similar for both treatments (Fig. 1, C). Light increased penetration in both control and EPTC-treated leaves (Fig. 1, D) but penetration was greatest in EPTC-treated plants, in both light and dark.

Concentration response. Increasing concn of EPTC resulted in a decrease in epicuticular wax deposition (Table 2). There was an inverse relationship between cuticular transpiration and wax levels. Penetration of ^{14}C-NAA

Figure 1. Dynamics of ^{14}C-NAA penetration into developing leaves of control and EPTC-treated plants. A. Time-course of penetration through upper and lower leaf surfaces. B. Influence of NAA concn on penetration through upper leaf surface. C. Effect of temperature on penetration of NAA through upper leaf surface. D. Effect of light on penetration of NAA through upper leaf surface.

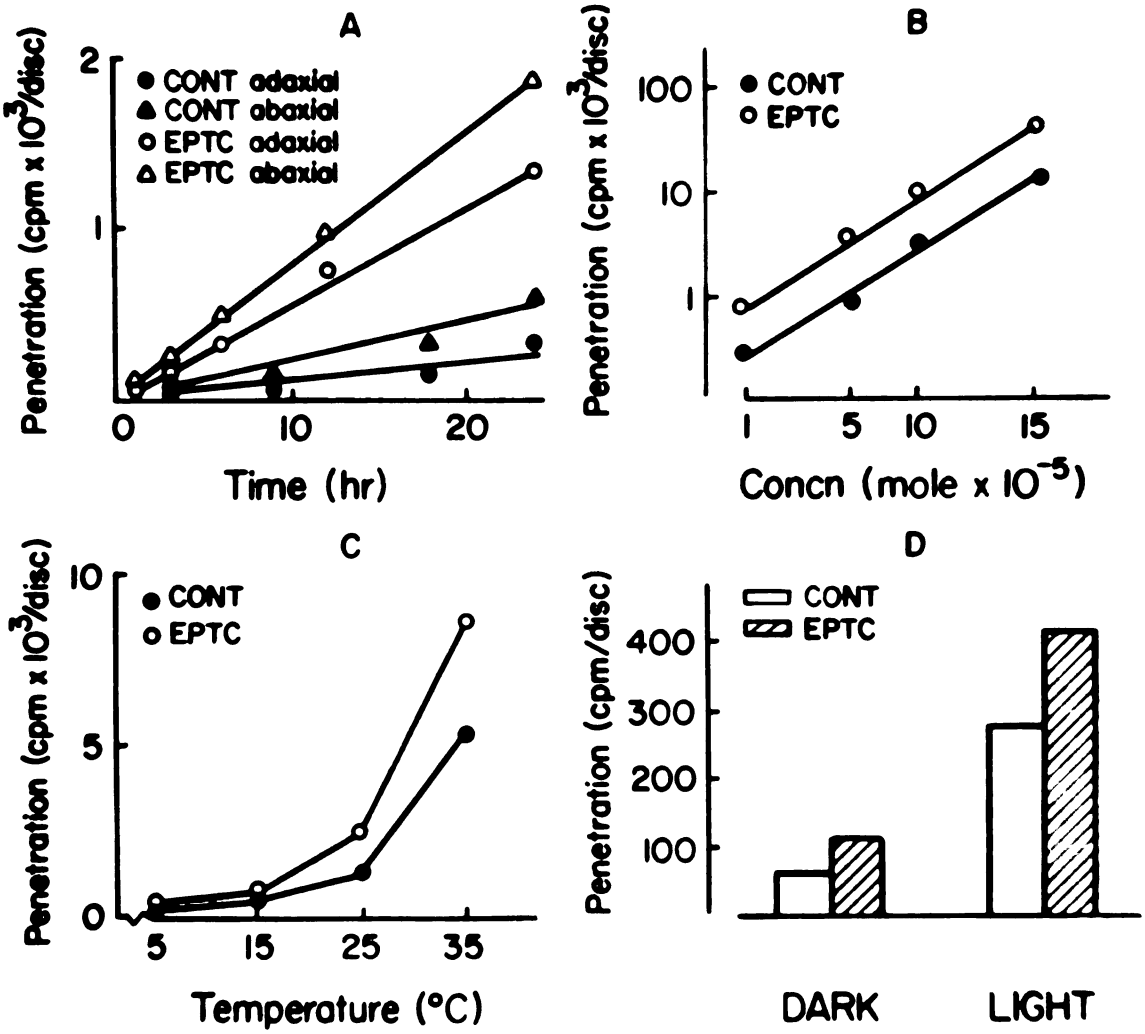

A
Penetration (cpm x 10³/disc)
2
1
0
10
20
Time (hr)
● CONT adaxial
▲ CONT abaxial
○ EPTC adaxial
△ EPTC abaxial
B
Penetration (cpm x 10³/disc)
100
10
1
1
5
10
15
Concn (mole x 10⁻⁵)
● CONT
○ EPTC
C
Penetration (cpm x 10³/disc)
10
5
0
5
15
25
35
Temperature (°C)
● CONT
○ EPTC
D
Penetration (cpm/disc)
400
300
200
100
CONT
EPTC
DARK
LIGHT

Table 2. The effect of EPTC concn on cuticular penetration, transpiration, and epicuticular wax deposition on developing leaves of cabbage.

EPTC[z] (kg/ha)	Penetration ^{14}C-NAA (cpm/disc)	Cuticular transpiration (mg/cm^2/20hr)	Epicuticular wax (µg/cm^2)
0.00	768a	3.5a	44.4a
0.28	1342b	3.9b	35.0b
0.55	1293b	4.8c	29.6c
1.10	1476b	5.5d	27.4c
2.20	1608b	6.9c	22.8d

[z]Mean separation within a column by Tukey's ω test, p = 0.05.

increased significantly due to EPTC application, there was a trend toward greater uptake with increasing concn of EPTC, but the increases among concn were not significant.

Duration of response. Uptake of ^{14}C-NAA was significantly greater in leaves isolated from the 7th node (in the bud stage at time of application) of EPTC-treated plants than corresponding leaves from control plants at all dates sampled. The magnitude of increase remained relatively constant (147%, at 7 days; 112%, at 42 days) with time. Permeability decreased in both treatments with time until 28 days after application of EPTC, then remained relatively constant until termination of the experiment at 42 days (Table 3).

Penetration of silver nitrate. More silver penetrated into leaves from plants treated with EPTC than in control plants, regardless of time (Fig. 2). Preferential reduction and accumulation of silver occurred first in the cuticular ledges of guard cells followed by the guard cells themselves, and then the anticlinal walls of the epidermal cells (Fig. 2).

Discussion

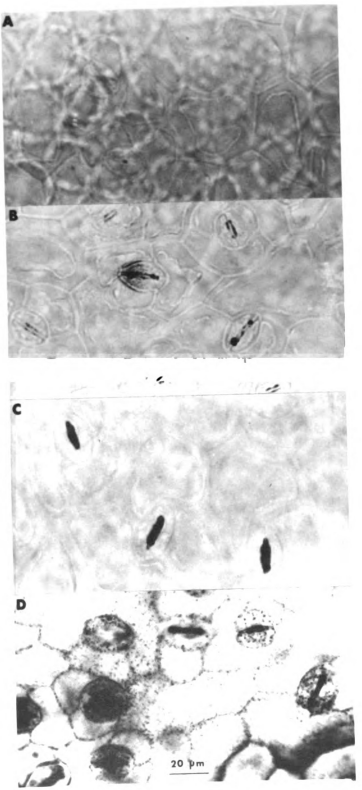
Cuticular membranes on leaves from EPTC-treated plants are more permeable than corresponding cuticular membranes on leaves of non-treated plants. Penetration of ^{14}C-NAA was greater into developing leaves of EPTC-treated cabbage,

Table 3. Effect of EPTC on penetration of ^{14}C-NAA into the leaf developing at the 7th node of cabbage plants as related to time after application.

^{14}C-NAA (cpm/disc) Penetration	Days after EPTC application[z] 14	21	28	35	42
Control	2392a	1279a	645a	715a	531a
EPTC	5912b	3127b	1114b	1250b	1126b
Increase (%)	147	144	73	75	112

[z]Mean separation within a column by Tukey's ω-procedure, p = 0.05.

Figure 2. Photomicrographs of leaf discs from leaves of non-treated and EPTC-treated cabbage plants following penetration of silver nitrate 0.1M, 0.01% X-77. A. Control, 15 min absorption. B. EPTC, 15 min absorption. C. Control, 60 min absorption. D. EPTC, 60 min absorption. Magnification as indicated.


A
B
C
D
20 µm

bean, and sugar beet than of non-treated plants and there was an inverse relationship between wax quantity and cuticular transpiration.

Penetration through the cuticle is thought to occur by diffusion (4). Based on studies of NAA penetration into pear leaves, Greene and Bukovac (14), concluded that uptake was controlled by both physical and metabolic factors. Data reported herein for time-course and concentration are linear (Fig. 1) for non-treated and EPTC-treated cabbage plants, which would support the concept that increased permeability after EPTC-treatment results from a reduction in those physical factors which control diffusion across the cuticular membrane. Metabolic factors which influence penetration and uptake of NAA are probably not affected by EPTC, as the magnitude of increased penetration due to treatment was similar in both the light and the dark (Fig. 1), and because of the linear relationship between penetration and time-course or concentration.

Juniper (19) suggested that increased plant injury to foliar applied herbicides was a consequence of greater retention, while others have implied that it results from greater permeability (3,6,13) or a combination of these factors. Retention is an important prerequisite to penetration, and before a biological response can be induced, the applied chemical must penetrate the cuticle and be transferred to an active site. Since diffusion across the cuticle is concentration dependent (Fig. 1) increased

retention, which influences dose, undoubtedly enhances penetration. However, in our study of cuticular transpiration where wettability and retention is not a factor, there is an inverse relationship between water loss and wax level, conclusively demonstrating an increase in cuticular permeability due to EPTC-treatment.

Data concerning the relationship between the quantity and chemical composition of epicuticular wax on cuticular permeability are not conclusive. Attempts have been made to relate quantity and quality to passage of water through artificial membranes impregnated with various epicuticular wax components (1,15). In these experiments, permeability to water is dependent upon the quantity of wax present until a certain threshold level is reached, after which increased deposition has little effect. When plated in equal quantities there was a differential influence on permeability due to the chemical composition of the wax.

Increasing concn of EPTC progressively decreased epicuticular wax levels on developing cabbage leaves (Table 2) but penetration of NAA was not correspondingly greater. If wax quantity has a significant influence on cuticular permeability an inverse relationship between wax level and penetration should exist. The absence of an inverse relationship between penetration of ^{14}C-NAA and wax level may result from the additional effect epicuticular wax fine-structure and chemistry have on wettability. Where wettability was not a factor, there was a significant

inverse relationship between cuticular transpiration and epicuticular wax level (Table 2).

Although epicuticular wax was not significantly reduced in bean or sugar beet (Table 1) mean wax levels declined as a result of EPTC application. An increase in penetration may have resulted from a compositional or structural change as well as a reduction in wax level on a plant that normally has a minimal amount of surface wax.

Silver nitrate penetration is increased by EPTC, and our observations indicate areas of preferential permeability (Fig. 2). Whether uptake of the undissociated NAA molecule follows the same pattern of uptake is not known. However, a reduction in epicuticular wax resulted in greater cuticular permeability for both the silver cation, and the undissociated NAA molecule.

Most experimental procedures employed to investigate the relationship between wax level and cuticular permeability involves manipulation of wax by: physical removal by brushing or with solvents (2,5); use of wax mutants (7); artificial membranes impregnated with wax; or by correlating wax levels from different species (20). These methods may result in assessments of permeability which are confounded by physical injury to the leaf surface, or by plants with different genetic background, which could erroneously influence the relationship between wax level and cuticular permeability. Chemical inhibition of wax deposition provides an alternate method of wax manipulation,

and may be a useful tool to assess the influence of wax on cuticular permeability.

Literature Cited

1. Baker, E. A., and M. J. Bukovac. 1971. Characterization of the components of plant cuticles in relation to the penetration of 2,4-D. Ann. Appl. Biol. 67: 243-253.

2. Bukovac, M. J. 1965. Some factors affecting the absorption of 3-chlorophenoxy-α-propionic acid by leaves of peach. Proc. Amer. Soc. Hort. Sci. 87: 131-138.

3. Cantliffe, D. J., and G. E. Wilcox. 1972. Effects of surfactant on ion penetration through leaf wax and a wax model. J. Amer. Soc. Hort. Sci. 97:360-363.

4. Currier, H. B., and C. D. Dybing. 1959. Foliar penetration of herbicides - Review and present status. Weeds 7:195-213.

5. Darlington, W. A., and J. Barry. 1965. Effects of chloroform and surfactants on permeability of apricot leaf cuticle. J. Agr. Food Chem. 13:76-78.

6. Davis, D. G., and K. F. Dusbabek. 1973. Effect of diallate on foliar uptake and translocation of herbicides in pea. Weed Sci. 21:16-18.

7. Denna, D. W. 1969. Transpiration and the waxy bloom in Brassica oleracea L. Aust. J. Biol. Sci. 23:27-31.

8. Dewey, O. R., P. Gregory, and R. K. Pfeiffer. 1956. Factors affecting the susceptibility of peas to selective dinitro-herbicides. Proc. 3rd. Brit. Weed Contr. Conf. 1:313-326.

9. _____, G. S. Harley, and J. W. G. MacLauchlen. 1962. External leaf waxes and their modification by root-treatment of plants with trichloroacetate. Proc. Roy. Soc. Ser. B. Biol. Sci. 133:532-550.

10. Flore, J. A., and M. J. Bukovac. 1974. Pesticide effects on the plant cuticle: I. Response of Brassica oleracea L. to EPTC as indexed by epicuticular wax production. J. Amer. Soc. Hort. Sci. 99:34-37.

11. _____. 1974. Pesticide effects on the plant cuticle: II. EPTC effects on the morphology and composition of Brassica oleracea L. leaf cuticle. J. Amer. Soc. Hort. Sci. (manuscript concurrently submitted).

12. _____. 1974. Pesticide effects on the plant cuticle. III. EPTC effects on the qualitative composition of Brassica oleracea L. leaf cuticle. J. Amer. Soc. Hort. Sci. (manuscript concurrently submitted).

13. Gentner, W. A. 1966. The influence of EPTC on external foliage wax development. Weeds. 14:27-31.

14. Greene, D. W., and M. J. Bukovac. 1972. Penetration of naphthaleneacitic acid into pear (Pyrus communis L.) leaves. Plant and Cell Physiol. 13:321-330.

15. Grncarevic, M., and F. Radler. 1967. The effect of wax components on cuticular transpiration -- model experiments. Planta. 75:23-27.

16. Holloway, P. J. 1969. Chemistry of leaf waxes in relation to wetting. J. Sci. Fd. Agr. 20:124-128.

17. Juniper, B. E. 1959. The effect of pre-emergent treatment of peas with trichloroacetic acid on the submicroscopic structure of the leaf surface. New Phytol. 58:1-5.

18. Kolattukudy, P. E., and L. Brown. 1974. Inhibition of cuticular lipid biosynthesis in Pisum sativum by thiocarbamates. Plant Physiol. 53:903-906.

19. Norris, R. F., and M. J. Bukovac. 1972. Influence of cuticular waxes on penetration of pear leaf cuticle by 1-naphthaleneacetic acid. Pest. Sci. 3:705-708.

20. _____. 1974. Penetration of 2,4-D in relation to cuticle thickness. Amer. J. Bot. 61:74-79.

21. Pfeiffer, F. K., O. R. Dewey, and R. T. Brunskill. 1959. Further investigation of the effect of pre-emergence treatment with trichloroacetic and dichloropropionic acids on the subsequent reaction of plants to other herbicidal sprays. Proc. 4th Int. Congr. Crop Protection. 1:523-525.

22. Steel, G. D., and J. H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill Book Co., Inc., New York.

23. Still, G. G., D. G. Davis, and G. L. Zander. 1970. Plant epicuticular lipids: Alteration by herbicidal carbamates. Plant Physiol. 46:307-314.

24. Wilkinson, R. E., and W. S. Hardcastle. 1969. EPTC effects on sickle pod petiolar fatty acids. Weed Sci. 17:335-337.

25. _____. 1970. EPTC effects on total leaflet fatty acids and hydrocarbons. Weed Sci. 18:125-128.

26. _____. 1974. Sicklepod surface wax response to photoperiod and S-(2,3-dichloroallyl)diisopropylthiocarbamate (Diallate). Plant Physiol. 53:269-275.

APPENDICES

Table A1. Abbreviations.

Abbreviation	Chemical name
Alar	Succinic acid-2,2-dimethylhydrazide
Avadex	S-2,3-Dichloroallyl diisopropylthiocarbamate (diallate)
Carbaryl	1-naphthyl N-methylcarbamate
CDEC	2-Chloroallyl diethyldithiocarbamate
Chlormequat	2-Chloroethyl-trimethyl ammonium chloride
Dieldrin	1,2,3,4,10,10-Hexachloro-exo-6,7,epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-exo-5,8-dimethalene
DNBP	2-sec-butyl-4,6-dinitrophenol
DNOC	4,6-dinitro-o-cresol
Diphenamid	N,N-dimethyl-2,2-diphenylacetamide
EPTC	S-Ethyl dipropylthiocarbamate
Folpet	N-trichloromethyl thiophyhalimide
GLC	gas-liquid chromatography
GC-MS	combined gas-liquid chromatography and mass spectrometry
MCPA	4-chloro-2-methylphenoxyacetic acid
MCPP	2-[(4-chloro-o-tolyl)oxy]propionic acid
MS	mass spectrometry
NAA	1-naphthaleneacetic acid
Paraquat	1,1-dimethyl-4,4-bipyridinium ion
Parathion	Diethyl 4-nitrophenyl phosphorothionate
TCA	trichloroacetic acid
TLC	thin-layer chromatography
2,4-D	(2,4-dichlorophenoxy)acetic acid
X-77	alkylarylpolyoxyethylene glycols, free fatty acids, and isopropanal

Table A2. The effect of leaf age on penetration and epicuticular wax deposition on leaves of cabbage.[1]

Leaf area	Penetration ^{14}C-NAA (cpm/disc)		Epicuticular wax ($\mu g/cm^2$)	
	Adaxial	Abaxial	Adaxial	Abaxial
13.2	1132	791	16.7	18.3
19.2	847	657	27.9	27.6
25.2	402	462	40.9	49.1
40.3	210	377	58.2	61.1

[1]Procedure: plant growth and EPTC-treatment (p. 6); epicuticular wax deposition (p. 7); and penetration (p. 54).

Table A3. Penetration, wettability, and epicuticular wax deposition on developing leaves of normal and glossy cultivars of cabbage as influenced by EPTC or TCA.[1]

Cultivar	Treatment	Penetration (cpm/disc)		Wettability ($^{0}\theta$)		Epicuticular wax ($\mu g/cm^2$)
		Adaxial	Abaxial	Adaxial	Abaxial	
Normal[2]	Control	143	242	146	144	67
	EPTC (2.2 kg/ha)	1031	1664	95	106	34
	TCA (11.0 kg/ha)	456	1187	134	140	52
Glossy[3]	Control	274	596	146	145	35
	EPTC (2.2 kg/ha)	1870	3464	117	118	20
	TCA (11.0 kg/ha)	1922	2582	119	115	26

[1]Procedure: plant growth and chemical application (p. 6); penetration (p. 54); epicuticular wax deposition (p. 7); and contact angle according to Mack, G. L. 1936. J. Phys. Chem. 40:159.

[2]Market Prize.

[3]B-1296.

Table A4. Effect of Cycloate and EPTC on epicuticular wax deposition, wettability, and ^{14}C-NAA penetration into sugar beet leaves.[1]

Measurement[2]	Control	Ro-Neet	EPTC
Epicuticular wax ($\mu g/cm^2$)	5.35a	6.12a	6.60a
Contact angle ($^0\theta$)	99.4a	96.1a	94.5a
Penetration ^{14}C-NAA (cpm/disc)	990a	1049a	2338b

[1]Procedure: plant growth and chemical application (p. 6); epicuticular wax deposition (p. 7); and penetration (p. 54).

[2]Mean separation by Tukey's ω-procedure, P = 0.05.

Table A5. Influence of treating solution pH on uptake of ^{14}C-NAA by developing cabbage leaves excised from control and EPTC-treated plants.[1]

pH	Uptake (cpm/disc)		
	Control	EPTC	Increase (%)
3.0	866	1538	78
4.0	356	1248	250
5.0	183	506	177
6.0	91	113	24
7.0	48	90	87

[1]Procedure: plant growth and EPTC treatment (p. 6); penetration (p. 54); pH adjusted with phosphate citrate buffer.

Table A6. Specifications of pesticide treating solutions used in penetration experiment.

Chemical	Molecular wt.	Specific activity	pH	Molarity
Dieldrin	383	72.4	5.3	2.6×10^{-7}
Paraquat	257	14.7	5.0	3.8×10^{-5}
Sevin	201	26.4	5.4	7.5×10^{-6}
2,4-D	222	29.0	5.2	4.0×10^{-5}
Diphenamid	239	00.8	5.4	2.2×10^{-6}
Calcium chloride	111	28.4	5.0	4.1×10^{-5}

Table A7. Penetration of foliar applied chemicals into leaves excised from control and EPTC-treated cabbage plants.[1]

Chemical	Relative partition value[2]	Penetration (cpm/disc) Control	Penetration (cpm/disc) EPTC	Penetration (% of applied) Control	Penetration (% of applied) EPTC	Penetration (% of applied) Increase
Dieldrin	99.0	138	147	91	96	6
Sevin	98.0	1549	2200	34	49	42
Diphenamid	52.4	170	661	.04	0.1	289
$CaCl_2$	0.3	11442	36721	.04	0.1	221
Paraquat	0.3	839	3873	.006	.03	361
2,4-D	0.6	368	2993	.001	.008	444

[1]Procedure: plant growth and EPTC-treatment (p. 6); penetration (p. 54); no buffer.

[2]Based on partitioning between equal volumes of water and chloroform, 100 = 100% in chloroform.

Table A8. The effect of surfactant (X-77) on penetration of ^{14}C-NAA, wettability, and retention, by leaves from control and EPTC-treated cabbage plants.[1]

Measurement	Treatment	Surfactant concentration (%) 0.00	0.01	0.10	1.00
Penetration (cpm/disc)	Control	447	1118	1392	1762
	EPTC	1524	6174	5363	4500
Wettability (0θ)	Control	141	111	60	49
	EPTC	115	70	51	41
Retention ($\mu l/cm^2$)	Control	1.8	56.1	51.1	47.1
	EPTC	23.3	52.6	49.5	41.6

[1]Procedure: plant growth and EPTC-treatment (p. 6); penetration modified by addition of surfactant (p. 58); wettability (p. 77); retention, ^{14}C-NAAm pH 3.2, .01 µc/ml, using method of Schonherr, J. 1969. Foliar penetration and translocation of succinic acid 2,2-Dimethylhydrazide (SADH). M.S. Thesis, Mich. State Univ., E. Lansing, 213 p.

Table A9. Penetration of ^{14}C-NAA into agar blocks through stomatous cuticular membranes isolated from developing leaves of control and EPTC-treated cabbage plants.[1]

Leaf surface	Penetration (cpm/disc) Control	EPTC
Adaxial	45	156
Abaxial	73	373
Adaxial minus epicuticular wax	1410	1392

[1]Procedure: plant growth and EPTC-treatment (p. 6); cuticle isolation (p. 8), modified by affixing glass vials to the leaf surface with rubber cement before enzymatic isolation. Penetration determined from activity in agar block.

Table A10. Penetration of ^{14}C-NAA into distilled water through stomatous cuticular membranes isolated from developing leaves of control and EPTC-treated cabbage plants.[1]

Time (hr)	Penetration (cpm/disc)			
	Adaxial		Abaxial	
	Control	EPTC	Control	EPTC
1	10	150	48	180
3	15	222	92	263
9	32	311	144	314
24	91	419	281	434

[1]Procedure: plant growth and EPTC-treatment (p. 6); cuticle isolation (p. 8), modified by affixing glass vials to the leaf surface with rubber cement before enzymatic isolation. Penetration determined from activity in distilled water, after diffusion from vial through stomatous cuticle.

Table All. Effect of EPTC-treatment on surface and permeability characteristics of cabbage leaves.[1]

Measurement	Ratio $\frac{\text{Abaxial}}{\text{Adaxial}}$		Ratio $\frac{\text{EPTC}}{\text{Control}}$	
	Control	EPTC	Adaxial	Abaxial
Penetration	1.99	1.17	6.29	2.55
Wettability	0.99	1.04	0.79	0.80
Epicuticular wax	1.12	1.03	0.41	0.44
Stomate density	1.27	1.32	0.99	1.01

[1]Procedure: plant growth and EPTC-treatment (p. 6); penetration (p. 54); wettability (p. 77); epicuticular wax deposition (p. 7); stomate density determined from silicone rubber impressions. Summary of several experiments.

Table A12. Hydrogen flame ionization detector response based on n-octocosane.[1]

Standard	Detector response
Alkane C-28	1.00
Aldehyde C-18	0.99
Primary alcohol C-26	0.95
Secondary alcohol C-29	0.86
Methyl ester of acid C-22	0.85
Ester C-36	0.82
Ketone C-35	0.67

[1]Column conditions: 1.25% SE-30 on Chromosorb W 80/100 mesh, nitrogen flow 40 ml/min, inlet and detector temp 360°C, column programmed 6°C/min, 120-350°.

Table A13. A compilation of the effects of EPTC on cabbage leaf cuticle. 0, no change; +, increase; -, decrease.

Characteristic	Alteration	Characteristic	Alteration
Quantitative		Cuticular permeability	
Cuticular membrane	0	^{14}C-NAA	+
Epicuticular wax	-	^{14}C-Dieldrin	0
Cutin composition	0	$AgNO_3$	+
Cuticle - other	+	Cuticular transpiration	+
		^{14}C-NAA + X-77	+
Qualitative		Wettability	
Epicuticular wax	+	Contact angle	+
Cuticular wax	+	Retention	+
Cutin composition	0	Retention + X-77	0
Morphology		Surface response	
Plant growth	0	Epi-wax, quantity	0
Leaf area	0	Epi-wax, quality	0
Surface fine-structure	-	Wettability	0
Cuticular membrane	0	Surface fine-structure	0
		Cuticular membrane thickness	0
		Uptake of ^{14}C-NAA	+

Table A14. Percent[1] composition of epicuticular wax isolated from Brassica oleracea L.

Chemical class	Chain length	Reference[2]			
		Purdy & Truter[3]	Macey & Barber[4]	Baker[5]	Flore & Bukovac[6]
Alkanes	total	-	33	45	26
	27	-	1	tr	tr
	28	-	1	tr	.4
	29	-	90	90	96
	30	-	2	1	.4
	31	-	5	8	2.3
Esters	total 40-48	12.6	-	2	8.3
Ketones	total 29	13.8	20	22	29
Aldehydes	total	-	2-5	4	5.5
	26	-	10.2	-	23.6
	27	-	tr	1	11.5
	28	-	49.3	17	25.3
	29	-	1.6	7	16.4
	30	-	36.1	74	20.0
sec.-alcohols	total 29				
Ketols	total	0.9	-	-	2.2
p-alcohols	total	8.7	-	5	7.6
	18	24	-	-	-
	20	6	-	-	-
	22	12	-	-	-
	24	6	-	30	6.6
	26	26	-	13	30.2
	28	6	-	2	17.1

Table A14. (cont'd.)

Chemical class	Chain length	Reference[2]			
		Purdy & Truter[3]	Macey & Barber[4]	Baker[5]	Flore & Bukovac[6]
Fatty acids	total	-	9	4	3.9
	14	-	7	-	5
	15	-	6	3	-
	16	-	1	28	2.5
	18	-	1	5	-
	20	-	-	5	-
	24	-	2	-	-
	25	-	1	5	-
	26	-	14	5	33
	27	-	3	-	-
	28	-	26	17	36
	29	-	5	6	-
	30	-	30	30	7.7

[1]Percent expressed as percentage of total wax, and as percentage of each class.

[2]References.
Baker, E. A. 1972. Msc. Thesis, Univ. Bristol. 134 p.
Flore, J. A., and M. J. Bukovac. 1974. See p.
Macey, M. J. K., and H. N. Barber. 1970. Phytochem.
Purdy, J. S., and E. V. Truter. 1963. Proc. Royal Soc. Ser. B Biol. Sci. 158:536.

[3]By TLC, var capitata cv Winninstadt, growing conditions not reported.

[4]By TLC, GLC var not reported, growing conditions not reported.

[5]By GLC, var gemmifera, "Cambridge Special" X "Ashvills strain" $F_7$7797, growing conditions: temp 21°C, RH 70%, radiant energy rate Wm^{-2} 80.

Table A15. Documentation of pesticide effects on the plant cuticle. 0, no effect; +, increase; -, decrease.

Reference[1]	Chemical	Plant	Effect
Baker et al, 1968	Chlormequat	blackcurrent	- wax
	Alar	blackcurrent	+ wax
Barner & Roder, 1962	Folpet	apple	+ cuticle thickness
Batt & Martin, 1961	phenylmecuric-acetate	apple fruit	- cutin
Cantliffe & Wilcox, 1972	EPTC	cabbage	+ Mn absorption - wax
Davis & Dusbabek, 1973	Diallate	pea	+ uptake 2,4-D + uptake atrazine + uptake TCA + uptake Diaquat
Dewey et al, 1956	TCA	Stellaria Veronica Chenopodium Gallum	+ DNBP injury
		pea	- wax + DNBP injury + spray retention
Dewey et al, 1962	TCA	pea kale	- wax
Flore & Bukovac, 1974	EPTC	cabbage	- wax
Gentner, 1966	EPTC	cabbage	- wax + DNBP injury + wettability + transpiration + fungal injury

Table A15. (cont'd.)

Reference[1]	Chemical	Plant	Effect
Juniper, 1959	TCA	pea	- wax fine structure + wettability
Kolattukudy & Brown, 1974	EPTC CDEC Avadex	pea	- wax - alkanes - sec-alcohol + esters
Pfeiffer, 1959	TCA	pea kale	+ DNBP injury + MCPA injury + MCPP injury + retention + transpiration
Still et al, 1970	Diallate	pea	- wax - p-alcohol
	CIPC	pea	0 wax[2]
	CDEC	pea	0 wax
Wilkinson, 1974	Diallate	sicklepod	- wax + fatty alcohol
Wilkinson & Hardcastle, 1969, 1970	EPTC	sicklepod leaf	- cuticle[3] + fatty acid
		sicklepod petiole	- cuticle[3]

[1]Baker, E. A., D. J. Dawkins, and B. D. Smith. 1968. Rep. Agric. Hort. Res. Stn., Univ. Bristol for 1967. 116.
Barner, J., and K. Roder. 1962. Proc. 10th International Orthocide Conference, Belgrade.
Batt, R. F., and J. T. Martin. 1961. Rep. Agric. Hort. Res. Stn., Univ. Bristol for 1960. 111.
Cantliffe, D. J., and G. E. Wilcox. 1972. J. Amer. Soc. Hort. Sci. 97:360.
Davis, D. G., and K. F. Dusbabek. 1973. Weed Sci. 21:16.
Dewey, O. R., D. Gregory, and R. K. Pfeiffer. 1956. Proc. 3rd Brit. Weed Contr. Conf. 1:313.
Flore, J. A., and M. J. Bukovac. 1974. J. Amer. Soc. Hort. Sci. 99:34.
Gentner, W. A. 1966. Weeds. 14:27.

Table A15. (cont'd.)

Kolattukudy, P. E., and L. Brown. 1974. Plant Physiol. 53:903.
Pfeiffer, R. K., O. R. Dewey, and R. T. Brunskill. 1959. Proc. 4th Int. Congr. Crop Protection. 1:523.
Still, G. G., D. G. Davis, and G. L. Zander. 1970. Plant Physiol. 46:307.
Wilkinson, R. E. 1974. Plant Physiol. 53:269.
Wilkinson, R. E., and W. S. Hardcastle. 1969. Weed Sci. 17:335.
_____. 1970. Weed Sci. 18:125.

[2]Determined by SEM.

[3]Cuticle thickness.

Table A16. Major fragments and relative intensity of mass spectra[1] obtained from 10,16-dihydroxyhexadecanoate, bisTMSi ether, isolated from cutin of Brassica oleracea.[2,3]

m/e	Fragment	Relative intensity
73	$(CH_3)_3Si$	100
75	$(CH_3)_2SiOH$	48
89	$(CH_3)_3SiO$	4
129	$CH_2 = CHCHOSi(CH_3)_3$	27
273	$CH_3CO_2(CH_2)_8CHOSi(CH_3)_3$	33
275	$(CH_3)_3SiO(CH_2)_6CHOSi(CH_3)_3$	52
415	$M - OCH_3$	3
431	$M - CH_3$	
446	$(CH_3)_3SiO(CH_2)_6\underset{\displaystyle OSi(CH_3)_3}{\underset{\vert}{CH}}(CH_2)_8CO_2CH_3$	--[4]

[1]By GC-MS utilizing a LKB-9000 GC-MS, interfaced with a PDP 8/I computer, separation on a 1% SE-30 column, temperature programmed at 5°C/min, 120-280°C, elution at 185°C. Ion source 70.0 eV.

[2]Mass spectra of major cutin constituent isolated from EPTC-treated plants was identical.

[3]Mass spectra identical to that published by Eglinton, G., H. Hunneman, and A. McCormick. 1968. Org. Mass. Spectro 1:593-611.

[4]Not detected.

Figure A1. GLC standard curves, 1.25% SE-30 on Chromosorb W 80/100 mesh, nitrogen flow 40 ml/min, inlet and detector temp 360°C, column temperature programmed 6°C/min., 120-350°. T rel based on elution time of a known/elution time of an internal standard.

A. methyl esters of fatty acids, T rel based on C-22, $Y = 11.9X + 10.5$.

B. p-alcohols, T rel based on C-22, $Y = 15.1X + 11.0$.

C. esters, T rel based on C-32, $Y = 25.3X + 6.7$.

D. aldehydes, T rel based on C-26, $Y = 15.4X + 10.6$.

E. alkanes, T rel based on C-24, $Y = 12.2 + 10.2$.

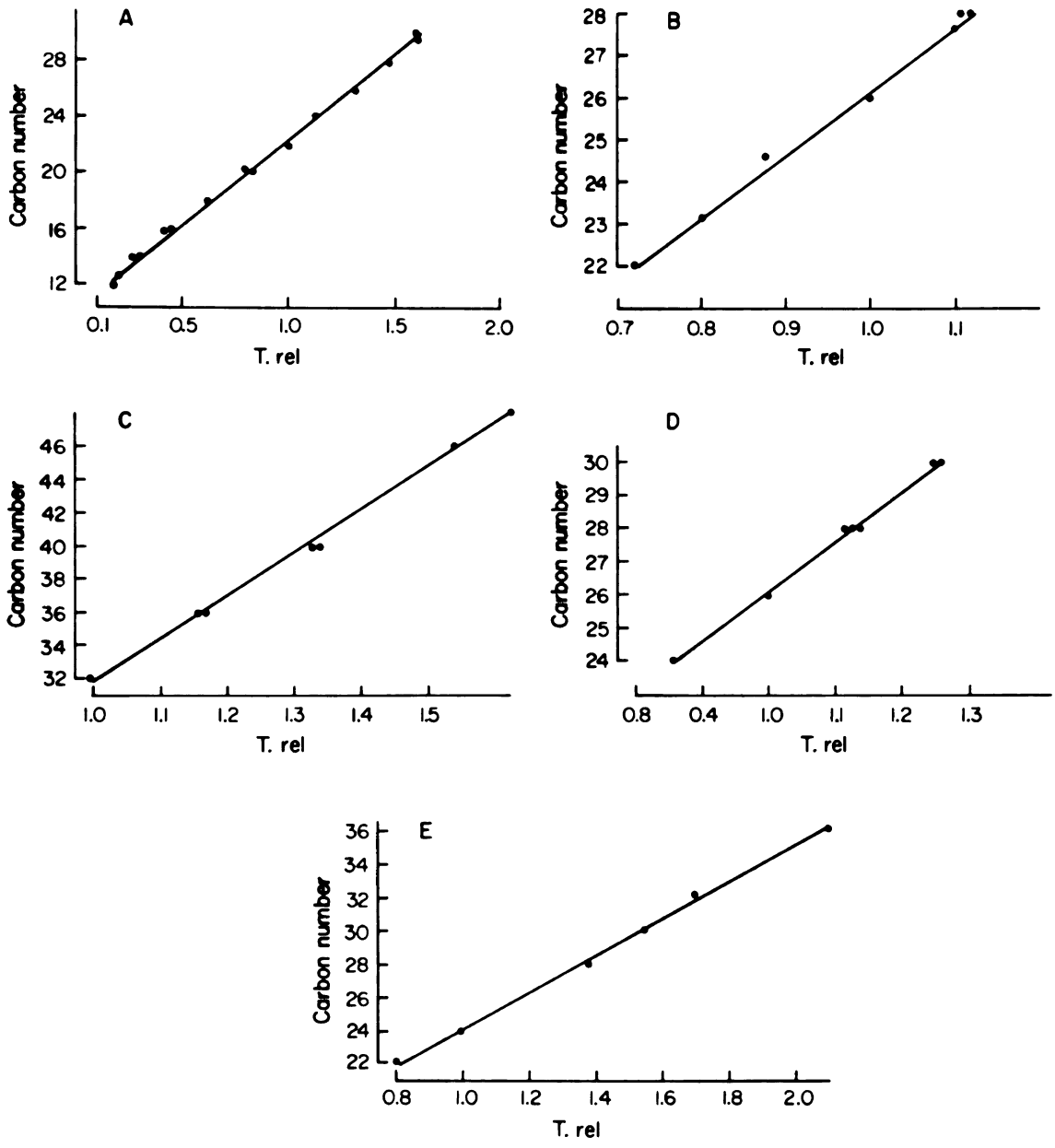

A
Carbon number
28
24
20
16
12
0.1
0.5
1.0
1.5
2.0
T. rel
B
Carbon number
28
27
26
25
24
23
22
0.7
0.8
0.9
1.0
1.1
T. rel
C
Carbon number
46
44
42
40
38
36
34
32
1.0
1.1
1.2
1.3
1.4
1.5
T. rel
D
Carbon number
30
29
28
27
26
25
24
0.8
0.4
1.0
1.1
1.2
1.3
T. rel
E
Carbon number
36
34
32
30
28
26
24
22
0.8
1.0
1.2
1.4
1.6
1.8
2.0
T. rel

Figure A2. The effect of leaf age on penetration of ^{14}C-NAA and epicuticular wax deposition on leaves of Brassica oleracea.

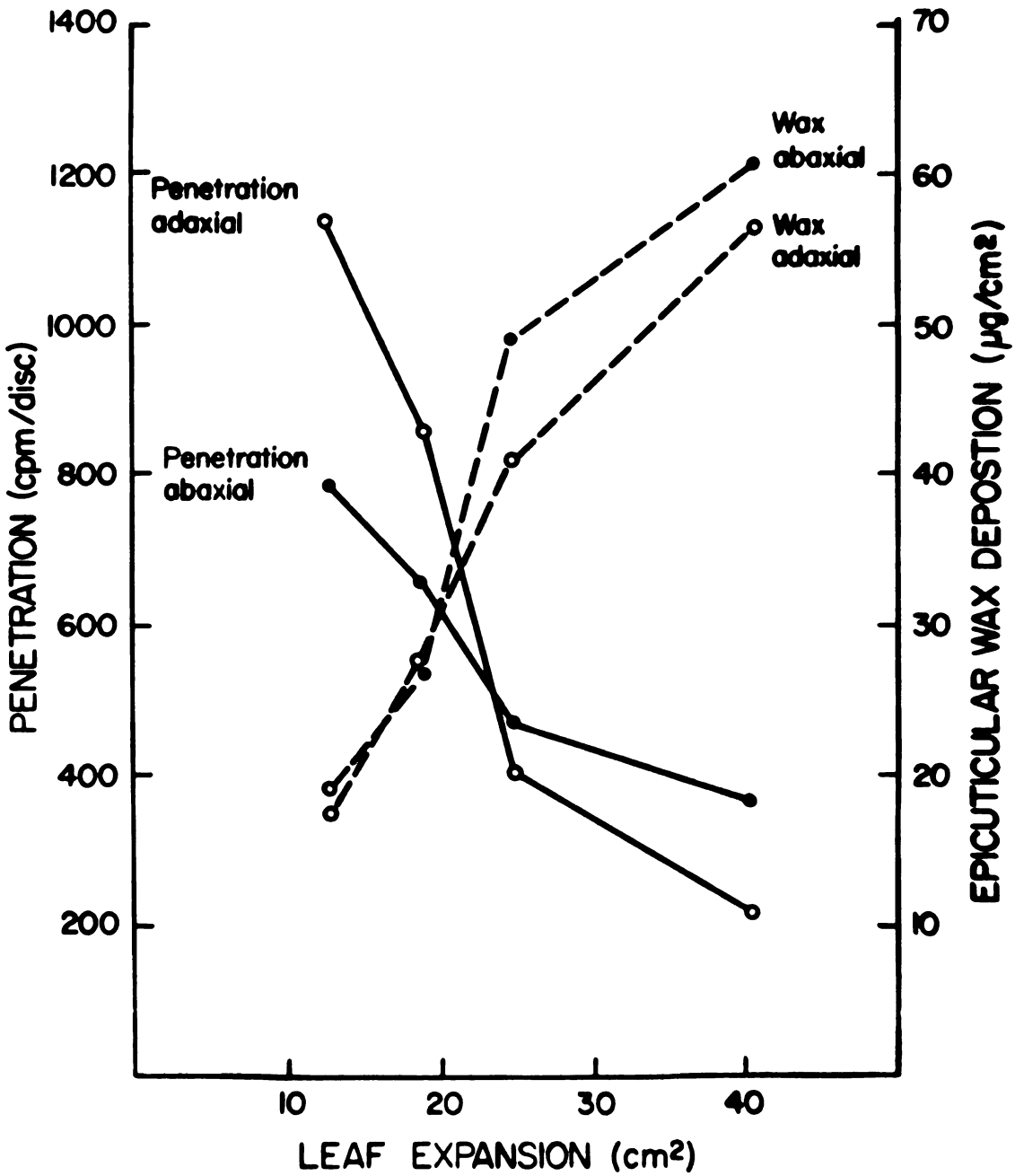
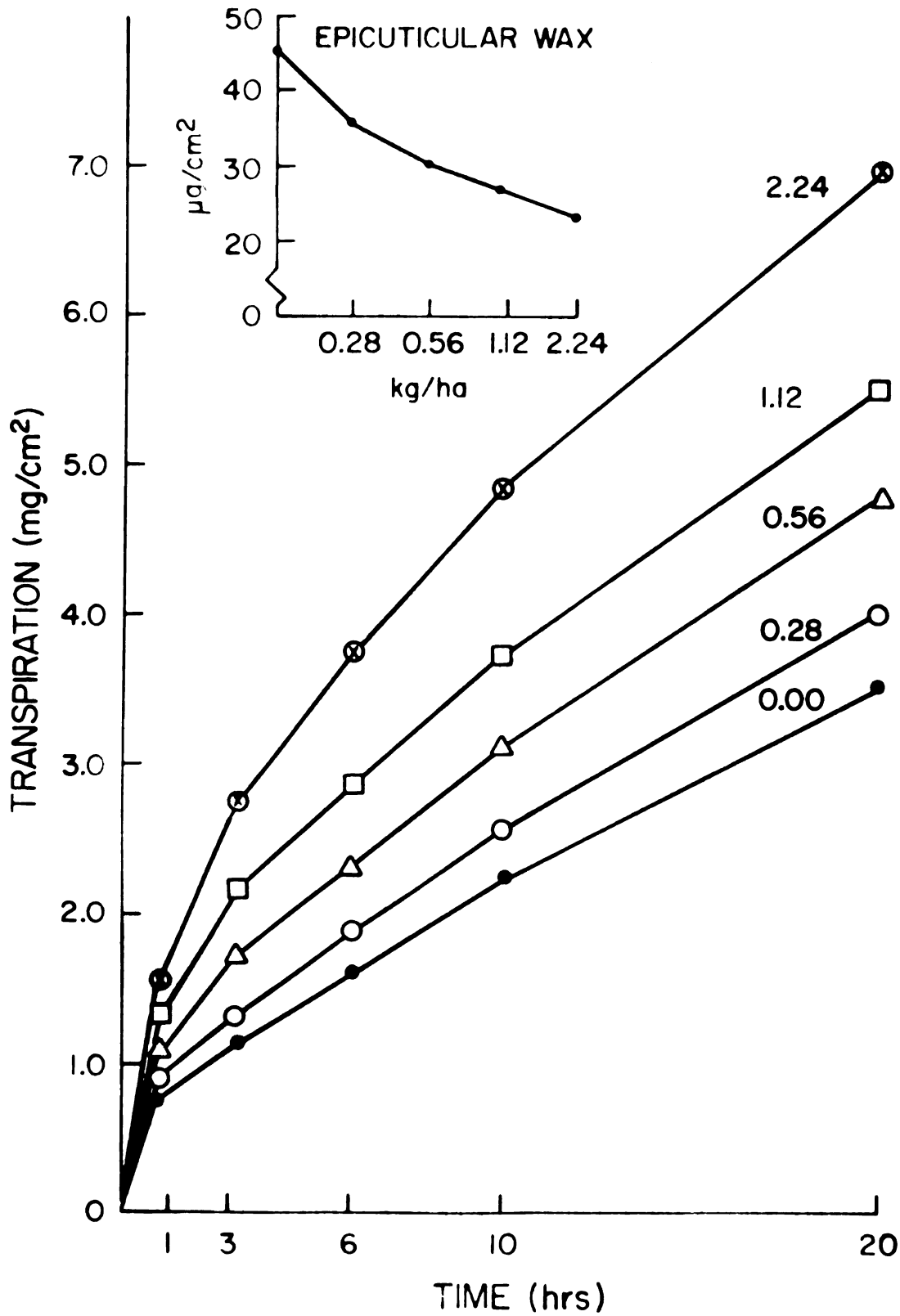

1400
1200
1000
800
600
400
200
70
60
50
40
30
20
10
Penetration adaxial
Penetration abaxial
Wax abaxial
Wax adaxial
PENETRATION (cpm/disc)
EPICUTICULAR WAX DEPOSTION (µg/cm²)
10
20
30
40
LEAF EXPANSION (cm²)

Figure A3. The effect of EPTC concn on cuticular penetration, transpiration, and epicuticular wax deposition on developing leaves of cabbage.


EPICUTICULAR WAX
μg/cm²
50
40
30
20
0
0.28 0.56 1.12 2.24
kg/ha
TRANSPIRATION (mg/cm²)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
2.24
1.12
0.56
0.28
0.00
1 3 6 10 20
TIME (hrs)