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A Mechanism of Chlorosis Caused by 1,3-Dimethyl-4-(2,4-dichlorobenzoyl)-5-hydroxypyrazole, a Herbicidal Compound[1]

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ABSTRACT

In organic solvents, 1,3-dimethyl-4-(2,4-dichlorobenzoyl)-5-hydroxypyrazole (DTP) converted chlorophyll *a* and *b* extracted from rice seedlings (*Oryza sativa* L. 'Kinmaze') into pheophytin *a* and *b*, respectively. On comparing the chlorophyll-converting activity of DTP to those of acetic, glycolic, 2,4-dichlorobenzoic, monochloroacetic, 2,6-dichlorobenzoic, pyruvic, and dichloroacetic acids, it was demonstrated that DTP induced H^+ into chlorophyll specifically. 5-Hydroxypyrazoles, which seem to be dissociable, converted chlorophyll into pheophytin *in vitro*. These compounds also induced chlorosis in sedge seedlings (*Cyperus serotinus* Rottb.), when the seedlings were grown in media containing these compounds. However, 5-hydroxypyrazoles, which seem to be undissociable, and analogs having no hydroxy group caused neither the chlorophyll conversion *in vitro* nor chlorosis in the seedlings. Chlorosis in barnyardgrass seedlings (*Echinochloa crus-galli* Beauv. var. *oryzicola Ohwi*) induced by DTP was reversed by cultivating the seedlings in media containing DTP plus NaOH, KOH, NH_4OH, $Ca(OH)_2$, sodium acetate, sodium pyruvate, sodium succinate, or sodium fumarate. Accumulation of the vinyl pheoporphyrin fraction in 4-day-old etiolated radish cotyledons (*Raphanus sativus* L. 'Minowase 2') was enhanced by incubating the cotyledons with δ-aminolevulinic acid in the dark. However, simultaneous treatment with δ-aminolevulinic acid and DTP reduced accumulation of the fraction and promoted formation of the uro, copro, and protoporphyrin fractions. These results suggest that DTP blocks the synthesis of protochlorophyllide in intact plants and induces consequent chlorosis, and the H^+-donating activity of DTP might cause the reduction of protochlorophyllide biosynthesis.

1,3-Dimethyl-4-(2,4-dichlorobenzoyl)-5-hydroxypyrazole is a herbicidal compound (18). The phytotoxic symptom of DTP[3] is chlorosis. The influence of chlorosis-inducing chemicals on plant pigment synthesis has been investigated. Amitrole showed influences on plastid pigments such as Chl and carotenoid (24). 4-Chloro-5-(dimethylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2H) pyridazinone (Sandoz 6706) markedly reduced Chl content in plants, and the Sandoz 6706-treated leaves had no spectrophotometric absorption band corresponding to Chl and carotenoid (15). Amitrole, dichlormate, and pyriclor inhibited normal carotinogenesis in etiolated wheat, and the phytotoxicity of these compounds was attributed to the inhibition of carotenoid synthesis which leads to subsequent photoreduction of Chl and chloroplasts. Sandoz 6706 and norflurazon also inhibited carotenoid synthesis, and the relationship between light intensity and the inhibition of carotenoid synthesis or the reduction of Chl content was investigated (2, 7). On the other hand, it has been concluded that amitrole did not block Chl synthesis in higher plants (5). Amitrole, dichlormate, and pyriclor caused no inhibition of Pchlide synthesis in wheat seedlings (4).

Preliminary studies on the mode of action of DTP showed the following: (*a*) DTP induced chlorosis in new tissues of plants without bleaching leaves already developed; (*b*) according to observations by light microscope, cells in bleached leaves of arrowhead, sedge, and barnyardgrass had a reduced number of chloroplasts and their color almost vanished; (*c*) green discs from mature leaves of wheat, barnyardgrass, corn, cucumber, and radish were bleached by keeping them in highly concentrated DTP solution. These observations suggest that DTP inhibits Chl biosynthesis and chloroplast development. Moreover, it seems that DTP is able to transform Chl directly. Here, we report Chl-transforming activity of DTP *in vitro* and an inhibitory effect of DTP on Pchlide biosynthesis, and we discuss the relationship between the *in vitro* action and chlorosis.

MATERIALS AND METHODS

Absorption spectra were recorded with a Hitachi 124 spectrophotometer.

Plant Materials. Rice seedlings (*Oryza sativa* L. 'Kinmaze') were grown on soil until the third leaf stage in a greenhouse. Barnyardgrass seeds (*Echinochloa crus-galli* Beauv. var. *oryzicola Ohwi*) previously dipped in water or 0.2 mM DTP solution for 24 h at 25 C were germinated and grown in chemical solutions in a greenhouse. Tubers of sedge (*Cyperus serotinus* Rottb.) with 0.5- to 1.0-cm buds were planted on 2-cm-thick agar (0.6%, w/v) in 4-cm diameter glass vials and 10 ml of a chemical suspension were added. Chemical suspensions were prepared by pulverizing chemicals with talcum powder and dissolving them in water with surfactants. A suspension without chemical was prepared as a control by the same method. The vials were placed in a greenhouse. Radish seeds (*Raphanus sativus* L. 'Minowase 2') were germinated and grown on moistened Vermiculite at 25 C in the dark.

Incubation. Seven g of etiolated cotyledons excised from 4-day-old radish seedlings were placed on a filter paper in a 9-cm-diameter Petri dish containing either 12 ml of water, 2 mM DTP, 10 mM ALA hydrochloride (Sigma), or 2 mM DTP plus 10 mM ALA hydrochloride at 25 C in the dark.

Preparation of Pigment Samples. Ten g of leaf blades from rice seedlings were homogenized with 100 ml of 80% aqueous acetone

[1] Research from a doctoral thesis submitted by K. K. to the Faculty of Agriculture, The University of Tokyo, Japan.

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[3] Abbreviations: DTP-Na: 1,3-dimethyl-4-(2,4-dichlorobenzoyl)-5-hydroxypyrazole sodium salt; ALA: δ-aminolevulinic acid; copro III TME: coproporphyrin III tetramethyl ester; proto IX DME: protoporphyrin IX dimethyl ester.

(v/v). The homogenate was filtered through four layers of gauze. The filtrate was washed with 40 ml of petroleum ether (boiling range 30–50 C). The ether fraction was evaporated to dryness and the resulting residue was dissolved in 40 ml of acetone. For extracting Chl *a*, *b* and pheophytin *a*, the above residue was chromatographed on silica gel thin layers. Bands of Chl *a*, *b* and pheophytin *a* were scraped from the plates and then each pigment was extracted with 30 ml of ethyl ether from the gel. One-third of the ether extract was used for recording spectra. The remaining ether extract was evaporated and dissolved in 4 ml of acetone. Column chromatography was also employed in preparing Chl *a*. The petroleum ether fraction from rice leaves was evaporated, the residue was redissolved in petroleum ether, the ethereal solution was chromatographed by a 3-cm-diameter column containing 20 g of silica gel 60 (Merck) which had been activated at 110 C for 1 h and equilibrated with petroleum ether. Solvents for elution were acetone-petroleum ether (1:3, v/v). The band of Chl *a* was separated and rechromatographed. After evaporating the solution of Chl *a* band, the residue was dissolved in 10 ml of acetone.

Addition of Chemicals to Pigments and Extraction of Pigment Derivatives. In Chl conversion tests, 5 mg of DTP were added to 2 ml of acetone solutions of Chl *a* or *b* prepared by TLC. The mixture was evaporated, and the residue was dissolved in 10 ml of ethyl ether for recording spectra. For HCl treatment, 1 ml of 0.1 N HCl was added to 2 ml of Chl *a* or *b* solutions and washed with petroleum ether. After evaporating the ether fraction, the residue was dissolved in 10 ml of ethyl ether for recording spectra. In examinations of pheophytin-forming activity, 2 ml of Chl *a* solution prepared by column chromatography were diluted with 10 ml of acetone and 4 ml of water. One ml of 0.01 M DTP, 0.01 M DTP-Na, or 0.01 M organic acids in acetone was added to the diluted Chl *a*. Half of the mixture was used for determining pH immediately and again 30 min after the addition. The remaining was mixed with 1 ml of 0.01 N NH_4OH 30 min after the addition, and then extracted with 10 ml of petroleum ether. This ether fraction was evaporated and separated by TLC. The band of pheophytin *a* was scraped and extracted with acetone. In addition to DTP analogs, 5 mg of each derivative were dissolved in 3 ml of acetone and 2 ml of water, and combined with 1 ml of pigment sample of rice seedlings. These mixtures were kept at room temperature for 5 h. Some of them were examined by TLC after being extracted with petroleum ether and subsequently evaporated.

Extraction of Porphyrins. Peroxide-free ethyl ether was used for extracting porphyrin and recording their spectra. Incubated radish cotyledons were ground with 60 ml of ethyl acetate-acetic acid (3:1, v/v) (10, 21). The homogenate was placed under 500 lux light for 30 min (21, 23). Extraction was conducted under the same light conditions, and followed by monitoring porphyrin fluorescence with a 366 nm UV lamp (6, 21). The homogenate was centrifuged. After removing the supernatant, the residue was washed twice with 15 ml of the same solvents. Uro-, copro-, proto-, and vinyl pheoporphyrins were fractionated by a modification of the method of Dresel and Falk (8) and Jones (17). The supernatants were combined and washed twice with 50 ml of saturated aqueous sodium acetate; the washings were back-extracted with 50 ml of ethyl acetate. This ethyl acetate fraction was combined with the supernatant and washed with 50 ml of 0.37 M sodium acetate. These aqueous sodium acetate extracts were reserved for extracting uroporphyrin. The combined ethyl acetate was washed with 2.7 N HCl to extract copro and protoporphyrin, and subsequently washed with 4.2 N HCl for extracting vinyl pheoporphyrin. The 2.7 and 4.2 N HCl extracts were independently adjusted to pH 4.0 with anhydrous sodium acetate and mixed with ether. The ether fractions were washed with water to remove acetic acid. Copro- and protoporphyrins were fractionated from the ether extract of 2.7 N HCl by 0.1 and 1.4 N HCl, respectively. These porphyrin extracts were used for spectrophotometric determination. The ether extract of 4.2 N HCl was evaporated and the residue was dissolved in dioxane to record spectra. In order to extract uroporphyrin, the combined aqueous sodium acetate was adjusted to pH 3.0 with concentrated HCl and washed with ethyl acetate. Uroporphyrin was extracted with 0.55 N HCl from the ethyl acetate fraction. This 0.55 N HCl extract was used for recording Soret region spectra.

TLC. For separating Chl *a*, *b* and pheophytin *a* or examining the pigment conversion, thin layers were prepared by spreading Silica Gel HF_{254} (Merck) on glass plates (20 × 20 cm) to an approximate thickness of 0.3 mm. Plates were air-dried and heated at 110 C for 1 h. Pigment residues dissolved in minimum acetone were developed with acetone-petroleum ether (boiling range 30–50 C) (1:3, v/v). For porphyrin separation, TLC aluminum sheet silica gel 60 (Merck) was purchased and activated as described above. Solvents were benzene-ethyl acetate-ethanol (4:1:1, v/v) (9, 21) or 2,6-lutidine-water (7:3, v/v) in NH_3 gas (19).

Spectrophotometric Determination. Chl content of barn-

Table I. *Action of DTP on Chl a and b*

Chl *a* and *b* in acetone were prepared from leaves of rice seedlings. DTP and 0.1 N HCl were added to the Chl *a* and *b* solution. After the addition, the pigments were extracted and dissolved in ether. Absorption spectra of the ethereal solutions were recorded.

Pigment	Absorption Maxima				
	nm				
Chl *a*	661	613	573	528	428
Chl *a* + DTP	667	607	530	502	407
Chl *a* + HCl	667	607	530	503	407
Pheophytin *a*	667	605	532	505	407
Chl *b*	642	592		452	
Chl *b* + DTP	653	598	556	518	432	410
Chl *b* + HCL	653	598	553	518	433	411
Pheophytin *b*[a]	655		550	520	434	413

[a] Absorption maxima reviewed by Goedheer (11).

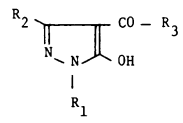
Table II. *Relationship between Acidity and Chl-converting Action of DTP*

Chl *a* in acetone-water (3:1, v/v) was prepared from leaves of rice seedlings and mixed with 0.01 M chemical solutions in acetone. The pH of the mixture was determined immediately and 30 min after the addition. Pheophytin *a* was extracted from the mixture 30 min after the addition and dissolved in acetone. The absorptions of the pheophytin *a* solutions at 666 nm were determined.

Added Chemical	pH of Mixture of Chl *a* and Chemical		Absorption Ratio of Pheophytin *a*
	0	30 min	
			%
Control	6.70	6.72	2.7
DTP	4.48	4.65	100
Acetic acid	5.98	5.98	5.2
Glycolic acid	4.86	4.90	6.1
2,4-Dichlorobenzoic acid	4.66	4.68	7.4
Monochloroacetic acid	4.32	4.40	10.6
2,6-Dichlorobenzoic acid	4.22	4.27	10.3
Pyruvic acid	3.90	3.93	19.7
Dichloroacetic acid	3.52	3.59	37.7
DTP-Na	7.69	7.60	6.2
Acetic acid + DTP-Na	6.67	6.60	8.2
Glycolic acid + DTP-Na	5.88	5.87	15.3
2,4-Dichlorobenzoic acid + DTP-Na	5.70	5.75	15.3
Monochloroacetic acid + DTP-Na	5.54	5.58	23.6
2,6-Dichlorobenzoic acid + DTP-Na	5.50	5.53	22.3
Pyruvic acid + DTP-Na	5.39	5.43	30.0
Dichloroacetic acid + DTP-Na	4.80	4.86	65.5

Table III. Relationship between chemical structure and Chl-converting or plant-bleaching activity.

Pigments in acetone were prepared from leaves of rice seedlings and combined with DTP derivatives solved in acetone-water (3:2, v/v). Some of the mixtures were examined on TLC. Tubers of sedge were grown in a greenhouse with the media containing these chemicals.

Added chemical: Chemical structure				Color change of pigment solution after addition	Chlorosis of sedge seedling concentration (mM)			
	R_1	R_2	R_3		0.5	0.1	0.05	0.01
1. 	CH_3	CH_3	CH_3	+*	+	+	-	-
	CH_3	CH_3	C_2H_5	+*	+	+	+	-
	CH_3	CH_3		+*	+	+	-	-
	CH_3	CH_3	OC_2H_5	+	+	-	-	-
	CH_3	CH_3	$OCH_2CH_2CH_3$	+	+	-	-	-
	CH_3	CH_3		+*	+	+	-	-
	CH_3	CH_3		+*	+	+	+	-
	CH_3	CH_3		+*	+	+	+	+
	CH_3	CH_3		+*	+	+	+	+
	CH_3	CH_3		+*	+	+	+	+
	H	CH_3		+	+	-	-	-
	CH_3	CH_3		+*	+	+	+	+
	C_2H_5	CH_3		+	+	+	+	+
		CH_3		+	+	+	-	-
		CH_3		+	+	+	-	-
		CH_3		+	+	+	-	-
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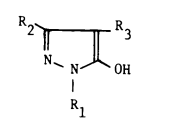
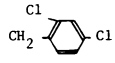
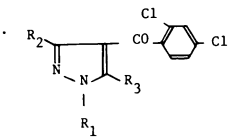
2. 	CH_3	CH_3	H	-	-	-	-	-
	CH_3	CH_3	CH_3	-	-	-	-	-
	CH_3	CH_3	$CH_2CH_2CH_2CH_3$	-	-	-	-	-
	CH_3	CH_3		-	-	-	-	-
	CH_3	CH_3		-	-	-	-	-
	CH_3	$COOCH_3$	H	-	-	-	-	-
	$-CH_2CH=CH_2$	CH_3	H	-	-	-	-	-
		CH_3		-	-	-	-	-
3. 	CH_3	CH_3	H	-	-	-	-	-
	CH_3	CH_3	Cl	-	-	-	-	-
	CH_3	CH_3	CH_3	-	-	-	-	-

+ : Color change of pigment solution or chlorosis of sedge seedling.
- : No variation of pigment solution or no chlorosis of sedge seedling.
* : Formation of pheophytins was proven by TLC.

yardgrass seedlings was calculated by the equation of Arnon (1) as follows. Shoots of 7-day-old seedlings were harvested, heated at 80 C for 3 min, ground with 80% aqueous acetone; the aqueous acetone was vacuum-filtered through a glass filter and examined for recording spectra. Absorptions of pheophytin *a* in acetone were measured at 666 nm. These absorbances were directly proportional to concentration of pheophytin *a*. In spectrophotometric determination of porphyrins, absorption spectra of each porphyrin fraction were recorded in the Soret region. Amounts of porphyrin were calculated by the following molar extinction coefficients: 4.89×10^5 at 399.5 nm for coproporphyrin (22), 2.75×10^5 at 408 nm for protoporphyrin (22), 1.93×10^5 at 419 nm for vinyl pheoporphyrin (12). To record visible region spectra, the copro- and protoporphyrin fractions were transferred to ether from their HCl solutions by adjusting the HCl extracts to pH 4.0. Standard porphyrins were obtained from copro III TME and proto IX DME (Sigma) as follows. The porphyrin esters were dissolved in 2.7 N HCl and kept for 1 to 16 h at room temperature, then the solutions were adjusted to pH 4.0 and extracted with ether; the ether extracts were washed with water.

RESULTS AND DISCUSSION

***In Vitro* Activity of DTP on Chl.** The Chl *a* and *b* extracted from rice leaves showed absorption maxima similar to those of Goedheer (11). The absorbances were converted into those of pheophytin, as extracted from control samples or reviewed by Goedheer (11), by adding DTP or HCl to the extracts (Table I). It is known that HCl removes Mg of Chl and subsequently forms pheophytin. These evidences suggest that DTP removed Mg from Chl *in vitro*. Since DTP is an acid (pKa = 4.1 ± 0.2) by virtue of the hydroxy group at the 5-position of the pyrazole ring, and since it had been demonstrated by TLC that DTP salts and esters caused no conversion of Chl, the acidity of DTP might be responsible for the removal of Chl-Mg.

Relationship between Acidity and Chl-converting Activity of DTP. In the Chl *a* solution, organic acids such as acetic, glycolic, 2,4-dichlorobenzoic, monochloroacetic, 2,6-dichlorobenzoic, pyruvic, and dichloroacetic acid converted Chl *a* into pheophytin *a* in proportion to their acidities. DTP exhibited approximately pH 4.5 in its mixture, equivalent to that of 2,4-dichlorobenzoic or monochloroacetic acid. However, the amounts of pheophytin formed by DTP were about 10 times more than by the acids. Even dichloroacetic acid, which showed the strongest acidity, could form merely one-third of the pheophytin formed by DTP. DTP-Na alkalized the Chl *a* solution and showed no Chl conversion. Additions of DTP to the Chl *a* plus organic acid solutions also increased pH in these mixtures. Nevertheless, the formation of pheophytin was raised markedly (Table II). These results indicate that DTP is not only an acid but also an agent introducing protons into Chl specifically. On the other hand, the chemical structure of DTP suggests that it might act as a chelator forming a stable complex with Mg. Konotsune (1976, unpublished) has proven, however, that the stability of DTP-Mg complex was no more than that of DTP-Na. It seems, therefore, that DTP does not function as a chelator for removing Chl-Mg.

Relationship between Chemical Structure and Chl-converting or Plant-bleaching Activity. The 5-hydroxypyrazoles which seem to be dissociable changed the green pigment solutions into brown colored mixtures. Some of them were examined by TLC and it was demonstrated that the color change was due to the formation of pheophytin in the mixtures. These compounds also induced chlorosis in the sedge seedlings. However, the hydroxypyrazoles, which seem to be undissociable, and the derivatives having no hydroxy group did not give rise to either the conversion of pigment or chlorosis in the plants (Table III). These observations indicate that the dissociable hydroxy group might be essential for inducing plant chlorosis. In addition, correspondence of the Chl conversion with the plant bleaching suggests that the induction of protons into Chl could be a mechanism of chlorosis by DTP.

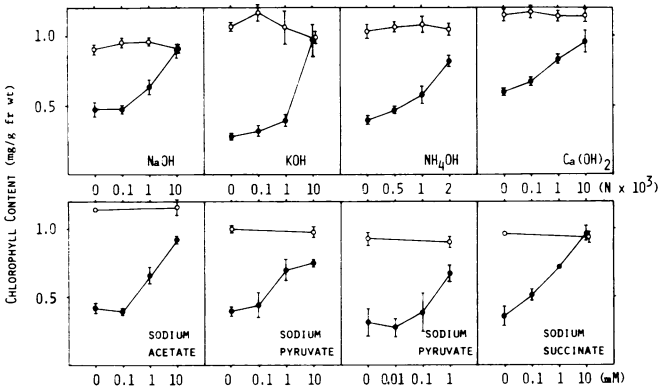


FIG. 1. Effect of alkalis and sodium salts of organic acid on chlorosis induced by DTP. Seeds of barnyardgrass were germinated in solutions of alkalis and sodium salts of organic acid with (●——●) or without 5 μM DTP (○——○).

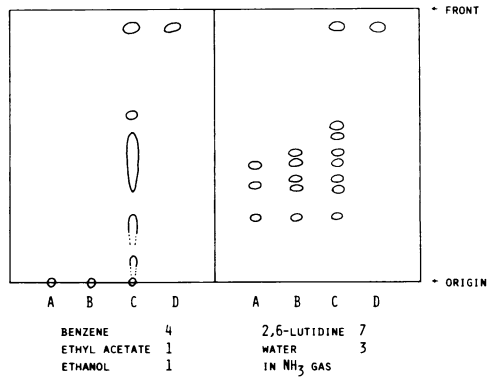


FIG. 2. Thin layer chromatograms of coproporphyrin fractions extracted from 4-day-old etiolated radish cotyledons incubated with 10 mM ALA plus 2 mM DTP in the dark for 5 h. Separation on TLC aluminum sheet silica gel 60. A: coproporphyrin fraction; B and C: copro III TME hydrolyzed by 2.7 N HCl for 2 h and 1 h, respectively; D: copro III TME.

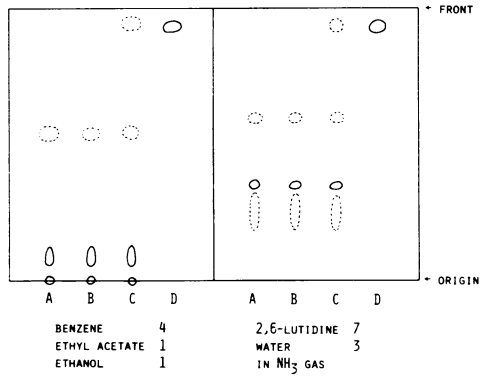


FIG. 3. Thin layer chromatograms of protoporphyrin fractions extracted from 4-day-old etiolated radish cotyledons incubated with 10 mM ALA or 10 mM ALA plus 2 mM DTP in the dark for 5 h. Separation on TLC aluminum sheet silica gel 60. A and B: protoporphyrin fraction from ALA- and ALA plus DTP-treated cotyledons, respectively; C: proto IX DME hydrolyzed by 2.7 N HCl for 2 h; D: proto IX DME.

Effect of Alkalis and Sodium Salts of Organic Acids on Chlorosis Induced by DTP. Chl content in barnyardgrass seedlings cultivated with DTP solution was reduced. Alkalis such as NaOH, KOH, NH_4OH, $Ca(OH)_2$, and sodium salts of organic acids such as acetic, pyruvic, fumaric, and succinic acid affected neither growth nor Chl content in the seedlings. However, when the plants were grown in simultaneous presence of DTP and one of these

alkalis or salts, the reduction of Chl content was reversed as the concentration of these chemicals increased (Fig. 1). The restorative effect on Chl content was also observed in cultivations with NaOH or sodium acetate by using barnyardgrass seeds previously dipped in DTP solution. These results suggest that the chemicals might block the activity of DTP. If DTP introduces protons into Chl in intact plants and leads to chlorosis, the addition of an alkali or a salt of organic acids might be inhibitory on the bleaching activity of DTP.

Effect of DTP on Biosynthesis of Chl Intermediates. ALA made etiolated radish cotyledons greenish during incubation in the dark. However, cotyledons incubated with ALA plus DTP became rather brownish. Thin layer chromatograms of the porphyrin extracts are shown in Figures 2 and 3. The coproporphyrin fraction from ALA plus DTP-treated cotyledons and copro III TME hydrolyzed by 2.7 N HCl for 2 h was not moved from the origin by benzene-ethyl acetate-ethanol (4:1:1, v/v). These coproporphyrins were separated into several spots in a similar manner by 2,6-lutidine-water (7:3, v/v) in NH_3 gas. The protoporphyrin fractions extracted from ALA- and ALA plus DTP-treated cotyledons also showed the same chromatographic behaviors as those of proto IX DME hydrolyzed for 2 h by either solvent system.

Some spectral properties of the porphyrin extracts and the standard porphyrins are given in Table IV. Absorption maxima of the coproporphyrin fraction were identical with those of hydrolyzed copro III TME. The protoporphyrin fractions were also similar in absorption properties to hydrolyzed proto IX DME. These chromatographic and spectral accordances indicate identity of the extracted porphyrin with the corresponding standard porphyrin. The vinyl pheoporphyrin fraction from ALA-treated cotyledons showed identical absorption maxima and absorption ratios except band IV with vinyl pheoporphyrin a_5 monomethyl ester reported by Granick (12). The vinyl pheoporphyrin fraction from ALA plus DTP-treated cotyledons exhibited different absorption ratios than those of the vinyl pheoporphyrin ester, although it had the identical absorption maxima with the porphyrin ester. Absorption spectra of the proto- and vinyl pheoporphyrins are shown in Figures 4 and 5.

Figure 6 shows Soret region absorption of the uroporphyrin fraction. No absorption peak was found in the fractions from control and DTP-treated cotyledons. However, the fractions from ALA- and ALA plus DTP-treated cotyledons had an absorption peak between 400 and 410 nm. The absorption intensity was enhanced by ALA plus DTP treatment. Uroporphyrin in 0.55 N HCl (2%, w/v) had its Soret maximum at 405 nm (8). It seems that these absorption peaks might be due to uroporphyrin, and DTP raised accumulation of uroporphyrin in cotyledons. Copro- and protoporphyrins were not detectable in control and DTP-treated cotyledons. ALA treatment slightly enhanced accumulation of these porphyrin fractions. However, the accumulation was obviously raised by ALA plus DTP treatment. On the contrary, the vinyl pheoporphyrin fraction was markedly accumulated by treatment with ALA alone (Table V). Time course experiments showed the same results as described above (Figs. 7 and 8); especially, amounts of the vinyl pheoporphyrin fraction were

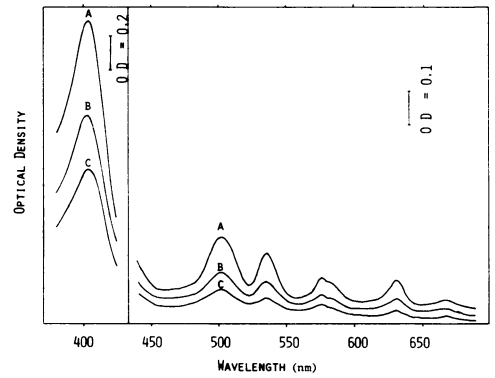


FIG. 4. Absorption spectra of protoporphyrin fractions in ether extracted from 4-day-old etiolated radish cotyledons incubated with 10 mM ALA or 10 mM ALA plus 2 mM DTP. OD: optical density; A: proto IX DME hydrolyzed by 2.7 N HCl for 16 h; B and C: protoporphyrin fraction from ALA plus DTP- and ALA-treated cotyledons, respectively.

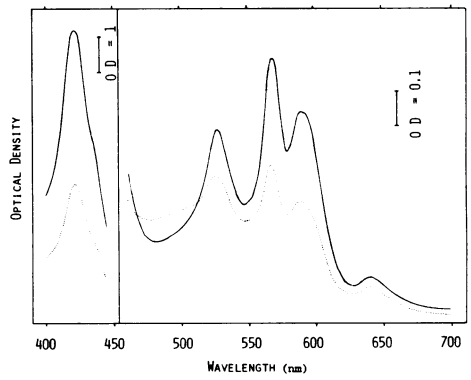


FIG. 5. Absorption spectra of vinyl pheoporphyrin fractions in dioxane extracted from 4-day-old etiolated radish cotyledons incubated with 10 mM ALA or 10 mM ALA plus 2 mM DTP in the dark for 5 h. OD: optical density; (——): vinyl pheoporphyrin fraction from ALA-treated cotyledons; (·····): vinyl pheoporphyrin fraction from ALA plus DTP-treated cotyledons.

Table IV. *Absorption Properties of Porphyrin Fractions Extracted from 4-Day-old Etiolated Radish Cotyledons Incubated with ALA or ALA Plus DTP in the Dark for 5 h*

Treatment	Absorption Maxima														
	Coproporphyrin (in ether)					Protoporphyrin (in ether)					Vinyl pheoporphyrin (in dioxane)				
	Band I	Band II	Band III	Band IV		Band I	Band II	Band III	Band IV		Band I	Band II	Band III	Band IV	
	nm														
ALA 10 mM						632	576	535	502	403	641	589	568	525	422
											(0.217	1.37	1.73	1.22	19.3)[a]
ALA 10 mM + DTP 2 mM	623	568	527	496	397	632	575	534	502	403	641	588	567	524	422
											(0.403	1.66	2.18	2.02	19.3)[a]
Standard porphyrin	623	568	526	496	396[b]	632	576	535	502	403[c]					
Reference	623	568	528	499	400[d]	634	577	536	504	405[d]	638	588	567	525	419[e]
											(0.215	1.40	1.77	0.825	19.3)[f]

[a] Absorption ratios at each maximum.
[b] Absorption maxima of copro III TME hydrolyzed by 2.7 N HCl for 2 h.
[c] Absorption maxima of proto IX DME hydrolyzed by 2.7 N HCl for 16 h.
[d] Absorption maxima reported by Rebeiz *et al.* (21).
[e] Absorption maxima reported by Granick (12).
[f] Molar extinction in dioxane × 10^{-4} reported by Granick (12).

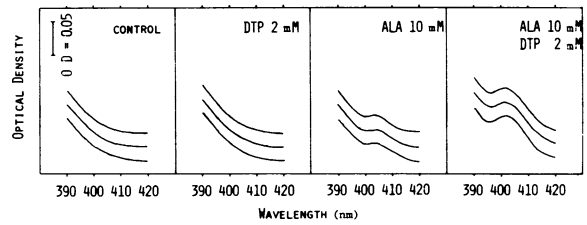


FIG. 6. Soret band region absorption spectra of uroporphyrin fractions in 0.55 N HCl quantitatively extracted from 4-day-old etiolated radish cotyledons incubated with chemicals in the dark for 5 h. OD: optical density. Determinations of three replications.

Table V. *Amounts of Copro, Proto, and Vinyl Pheoporphyrin Fractions Extracted from 4-Day-old Etiolated Radish Cotyledons Incubated with DTP, ALA, or ALA Plus DTP in the Dark for 5 h*

The data are the means of three replications and standard deviations.

Treatment	Fraction		
	Coproporphyrin	Protoporphyrin	Vinyl pheoporphyrin
		nmol/g fresh wt	
Control	ND[a]	ND	5.08 ± 0.12
DTP 2 mM	ND	ND	5.70 ± 0.22
ALA 10 mM	t[b]	1.29 ± 0.07	43.9 ± 3.1
ALA 10 mM + DTP 2 mM	0.39 ± 0.01	3.58 ± 0.36	19.7 ± 1.1

[a] Not detected.
[b] Trace amounts.

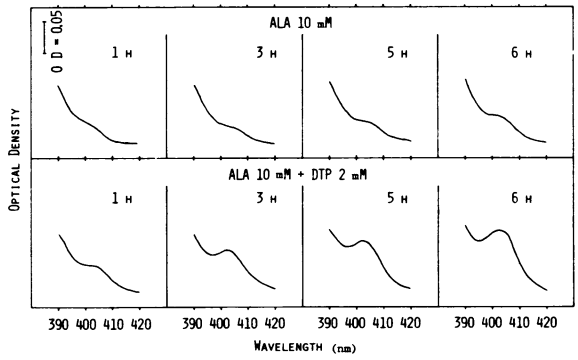


FIG. 7. Soret band region absorption spectra of uroporphyrin fractions in 0.55 N HCl extracted in the course of time from 4-day-old etiolated radish cotyledons incubated with ALA or ALA plus DTP in the dark. OD: optical density.

extremely reduced by ALA plus DTP treatment, whereas ALA sharply increased accumulation of the fraction (Fig. 8).

Many intermediates have been found in Chl biosynthesis (3, 14, 20). ALA promoted formation of Mg-porphyrins and Pchlide in etiolated barley seedlings (13) and cucumber cotyledons (21) in the dark. In this study, ALA made etiolated radish cotyledons greenish in the dark and sharply accumulated the vinyl pheoporphyrin fraction. Because of the close similarity in the spectrophotometric properties to vinyl pheoporphyrin a_5 monomethyl ester, and the solubility in 4.2 N HCl (15% w/v) which indicates lack of phytyl side chain (17), it is certain that the fraction consists of Mg-free derivative of Pchlide which had been formed by acid extraction. DTP inhibited accumulation of this fraction and promoted formation of the uro-, copro-, and protoporphyrin fractions in the simultaneous presence of ALA. These suggest that DTP caused the inhibition of Pchlide biosynthesis, which might lead to chlorosis in plants. However, it is uncertain where and how DTP blocked Pchlide biosynthesis. It has been proven that DTP had a specified potency introducing protons into Chl *in vitro*, and that the proton-donating potency correlated with plant chlorosis induced by DTP. In addition, Mg can be liberated from Mg-porphyrins by acid (16, 21). On these facts, it seems that the removal of Mg from Mg-porphyrins could be the mechanism by which DTP inhibits Pchlide synthesis. If DTP works so, chlorosis might be induced in new tissues of plants more effectively than in old leaves.

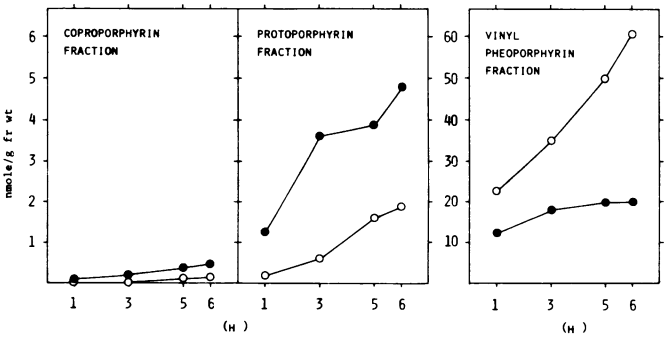


FIG. 8. Amounts of copro, proto, and vinyl pheoporphyrin fractions extracted in the course of time from 4-day-old etiolated radish cotyledons incubated with 10 mM ALA (○——○) or 10 mM ALA plus 2 mM DTP (●——●).

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