The Effects of Tentoxin on Chlorophyll Synthesis and Plastid Structure in Cucumber and Cabbage[1,2]

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ABSTRACT

To determine if chlorosis caused by tentoxin, a toxin produced by *Alternaria tenuis* Nees., is due to interference with chlorophyll synthesis directly or to disruption of normal chloroplast development, the effects of the toxin on these processes in cucumber (*Cucumis sativus* L.) and cabbage (*Brassica oleracea* L., var. capitata) were studied. Cucumber cotyledons are highly sensitive to the toxin but exhibited no interference with the conversion of protochlorophyll(ide) to chlorophyll(ide) or with the general time course pattern of chlorophyll synthesis, although there was a 90% reduction in chlorophyll concentration. In cabbage, which shows no chlorosis in the presence of the toxin, there was a slight stimulation of chlorophyll synthesis in the presence of the toxin. Electron microscopy revealed that in cucumber, toxin treatment interferes with development of prolamellar bodies and lamellae, and results in deformed plastids. No such effects were noted in toxin-treated cabbage tissues. Plastids in toxin-treated cotyledons of both cucumber and cabbage contained more starch than plastids in nontreated tissues. It was concluded that tentoxin acts through disruption of normal plastid development, rather than through direct interference with chlorophyll synthesis.

A chlorosis-inducing toxin (trivial name: tentoxin) produced by the fungus *Alternaria tenuis* Nees. has been characterized (8) as cycloleucyl-*N*-methyl alanylglycyl-*N*-methyl dehydrophenylalanyl. The toxin causes a sharply delimited, variegated, yellow chlorosis in germinating seedlings of many dicotyledonous species, but it has no apparent effect on tomato and members of the *Cruciferae* and *Gramineae* (1, 7). Templeton *et al.* (7) observed that cucumber seedlings were more sensitive to tentoxin in darkness than under continuous light, and that those exposed 48 to 50 hr after the initiation of germination were insensitive to the toxin. They suggested that activity of the toxin is associated with some step late in the development of chloroplasts. Saad *et al.* (4) found that the toxin was active at concentrations as low as 0.2 μg/ml on cucumber seedlings germinated under continuous light.

Templeton *et al.* (7) proposed that the toxin may: (*a*) interfere with chlorophyll synthesis but not with plastid development, or

(*b*) interfere with plastid development, thereby affecting chlorophyll synthesis indirectly. The present investigation was undertaken to determine if either of these hypotheses is correct and to determine if cellular organelles other than plastids are affected by the toxin. A preliminary report of this study has been published (2).

MATERIALS AND METHODS

Procedures for the production, purification, and quantitation of tentoxin have been described (4). Seeds of cucumber (*Cucumis sativus* L., c.v. SMR-18) and cabbage (*Brassica oleracea* L., var. capitata, c.v. Jersey Queen) were germinated in distilled water or tentoxin (30 μg/ml), in darkness, at 24 C, for 96 hr, unless otherwise noted. Seedlings were either harvested under a green safelight or exposed to 200 ft-c from General Electric Cool-white fluorescent lamps at 24 C.

Protochlorophyll and chlorophyll were extracted by homogenizing 1 g (fresh wt) of cotyledons in 50 volumes (w/v) of acetone in a Sorvall Omni-mixer. The acetone solution was centrifuged at 10,000g for 10 min and evaporated to dryness under reduced pressure. The residue was dissolved in anhydrous diethyl ether (5 ml/g of cotyledons), and the absorption spectrum from 550 to 720 mμ was measured in a Cary model 15 spectrophotometer. Concentrations of protochlorophyll and chlorophyll in ether solutions were estimated by the methods of Koski (3) and Smith and Benitez (5), respectively.

Chlorophyll synthesis was measured as follows: cotyledons were extracted as described above, but the dried residue was redissolved in 80% acetone rather than diethyl ether. Absorption spectra from 640 to 720 mμ were recorded, and total chlorophyll was estimated by the method of Vernon (9).

For electron microscopy, cotyledons were fixed in 5% glutaraldehyde, rinsed in cacodylate buffer, and postfixed in osmic acid (Palade's fixative) overnight. Tissue pieces from toxin-treated, light-grown, cucumber seedlings were cut from areas of cotyledons which appeared to be completely chlorotic, and those from light-grown control and dark-grown seedlings were cut from analogous portions of the cotyledons. The tissues were dehydrated in a graded acetone series, stained overnight with saturated uranyl acetate during the 70% acetone dehydration step, and embedded in araldite 6005. Post-section staining was done with lead citrate.

RESULTS

Conversion of Protochlorophyll(ide) to Chlorophyll(ide). Experiments with cucumber revealed somewhat less protochlorophyll in toxin-treated than in control cotyledons, but conversion to chlorophyll during a 15-min exposure to light was complete, as measured by loss of absorbance at 624 mμ, and additional chlorophyll synthesis occurred (Table I). Attempts to extract

protochlorophyll from etiolated cabbage cotyledons, even in complete darkness, were unsuccessful, but small amounts of chlorophyll were always present prior to exposure to light, at higher concentrations in toxin-treated cotyledons than in the controls. More chlorphyll was synthesized in toxin-treated than in control cotyledons upon exposure of cabbage seedlings to 15 min of light. Our ability to isolate protochlorophyll from cucumber seedlings but not from cabbage seedlings is in agreement with a report by Strain and Svec (6).

Time Course of Chlorophyll Synthesis. Control and toxin-treated cucumber seedlings exhibited similar sigmoid patterns of chlorophyll synthesis during a 24-hr period of exposure to light (Fig. 1). However, toxin-treated seedlings synthesized chlorophyll

Table I. *Effects of Tentoxin on Production of Protochlorophyll(ide)
and Chlorophyll(ide) in Cucumber and Cabbage and on
Light-induced Conversion of Protochlorophyll(ide)
to Chlorophyll(ide)*

Seedlings were germinated in darkness for 96 hr. Light treatments were exposed to 200 ft-c of light for 15 min prior to extraction.

Test Plant	Treatment		Pigment Concentration	
	Toxin	Light	Proto-chlorophyll	Chlorophyll
			μg/g fresh wt	
Cucumber	—	—	3.3	0.0
Cucumber	—	+	0.0	5.4
Cucumber	+	—	2.3	0.0
Cucumber	+	+	0.0	4.2
Cabbage	—	—	0.0	0.8
Cabbage	—	+	0.0	3.9
Cabbage	+	—	0.0	1.1
Cabbage	+	+	0.0	5.4

at a reduced rate and contained only about 10$\%$ as much chlorophyll as control seedlings after 24 hr of exposure to light. This value corresponds to the maximum attainable chlorosis (90$\%$) in toxin-treated cucumber seedlings germinated under continuous light (4). Treated cucumber seedlings germinated in darkness were uniformly pale green after 24-hr exposure to light, and they did not exhibit the variegated chlorosis which is characteristic of seedlings germinated under continuous light (4, 7).

Plastid Structure. The normal chloroplasts of cucumber cotyledons which developed under continuous light, in the absence of tentoxin, contained prolamellar bodies 96 hr after sowing (Fig. 2A). Granalamellae and intergranalamellae were well developed and quite regular in size and shape, and only small amounts of starch were present. Chloroplasts from cotyledons of toxin-treated seedlings, germinated under continuous light, had irregular shapes, many peripheral vesicles, and few or no granalamellae or intergranalamellae (Fig. 2B). Starch was evident in these chloroplasts.

Plastids from cucumber cotyledons which developed in the dark in untreated seedlings had large prolamellar bodies and little or no development of granalamellae or intergranalamellae (Fig. 2C). Vesicles were observed in these plastids. Toxin-treated cells from cotyledons developed in the dark contained plastids which were nearly filled with starch (Fig. 2D). Vesicle development and occasionally some lamellar development had occurred. The absence of prolamellar bodies in these plastids suggests a very low frequency of these bodies; however, the stroma in these plastids was very dense, and this may have obscured the prolamellar bodies.

The chloroplasts of treated and control cabbage cotyledons kept in the light showed normal development of granalamellae and intergranalamellae (Fig. 3, A, B). Packing of the granalamellae was not so tight and, in general, membranes were not so straight and closely appressed in toxin-treated tissue as in control tissue.

In control cabbage cotyledons kept in the dark (Fig. 3C) few
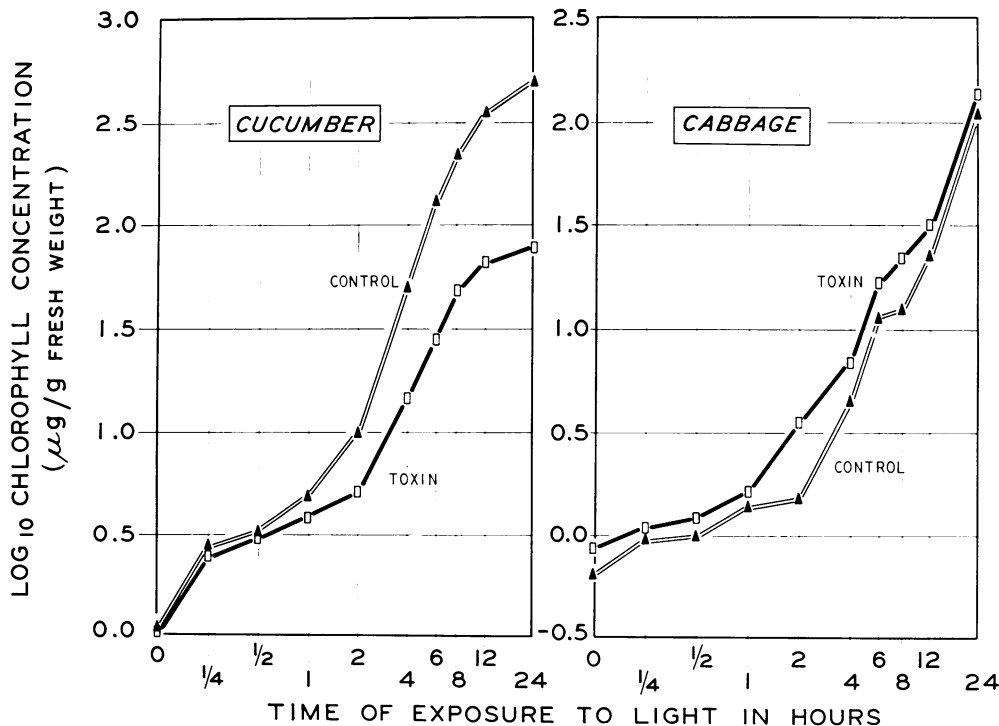

FIG. 1. Time course of chlorophyll synthesis in cotyledons of cucumber and cabbage as affected by tentoxin. Seedlings were germinated for 96 hr in darkness, in distilled water or in 30 μg/ml of tentoxin, prior to exposure to light.
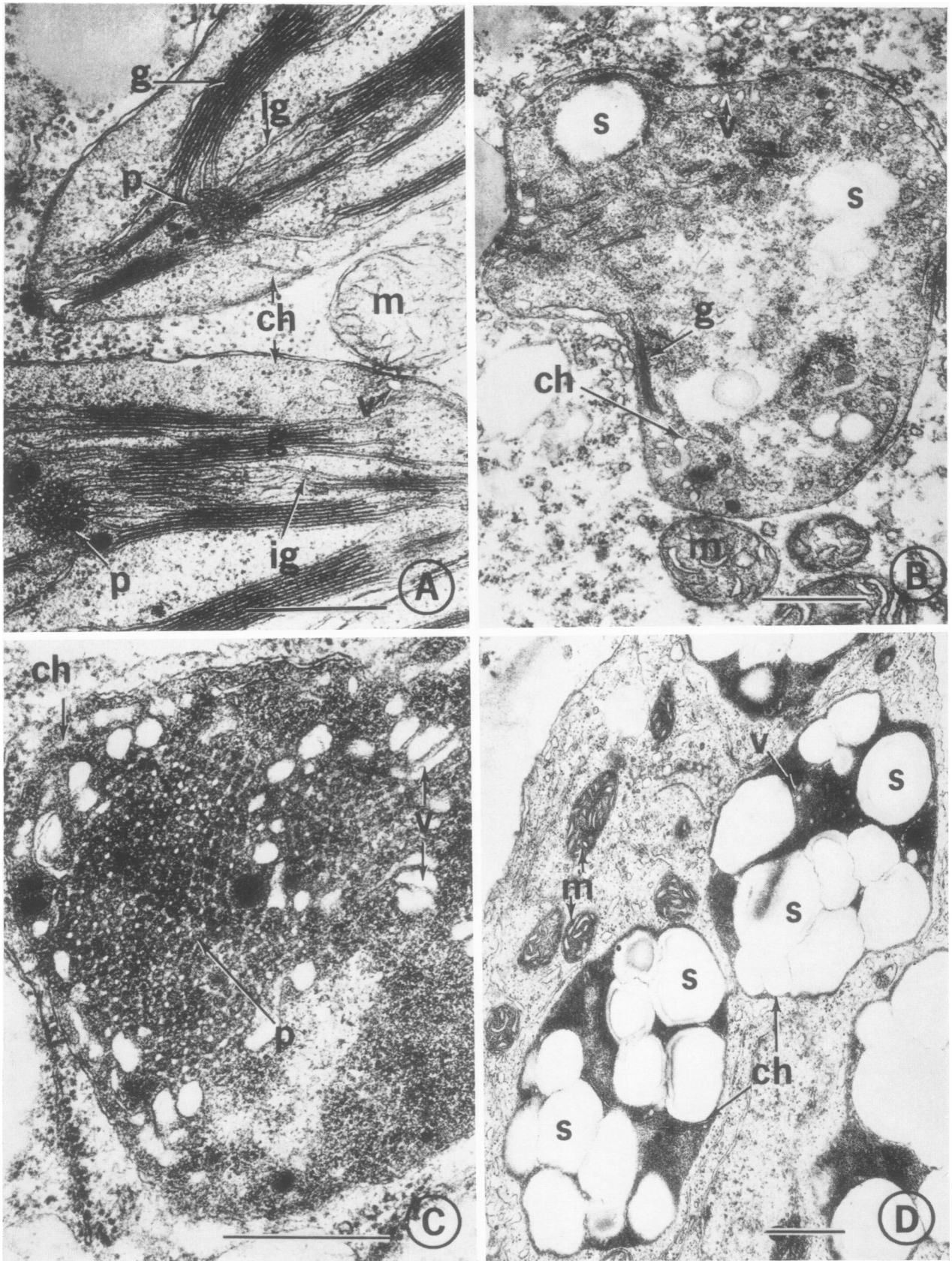
Fig. 2. Differential response of plastids in cucumber cotyledons to light and tentoxin treatments. A: Chloroplasts developed under a continuous light regime. Note the prolamellar bodies indicating juvenile plastids. × 52,000; B: The same as part A except for toxin treatment during germination. Note the complete disorganization and absence of a lamellar system, and the presence of starch grains. × 36,000; C: A plastid from cotyledon tissue not exposed to light during germination. Observe the large prolamellar body and the vesiculation. No lamellar structures have developed. × 60,000. D: The same as part C except for toxin treatment during germination. × 26,000. Note the predominance of the starch grains in the chloroplasts; ch: chloroplast; g: granalamellae; ig: integranalamellae; m: mitochondrion; p: prolamellar body; ph: phytoferritin; s: starch grain; v: vesicle. Solid lines at base of figures represent 0.5 μ.
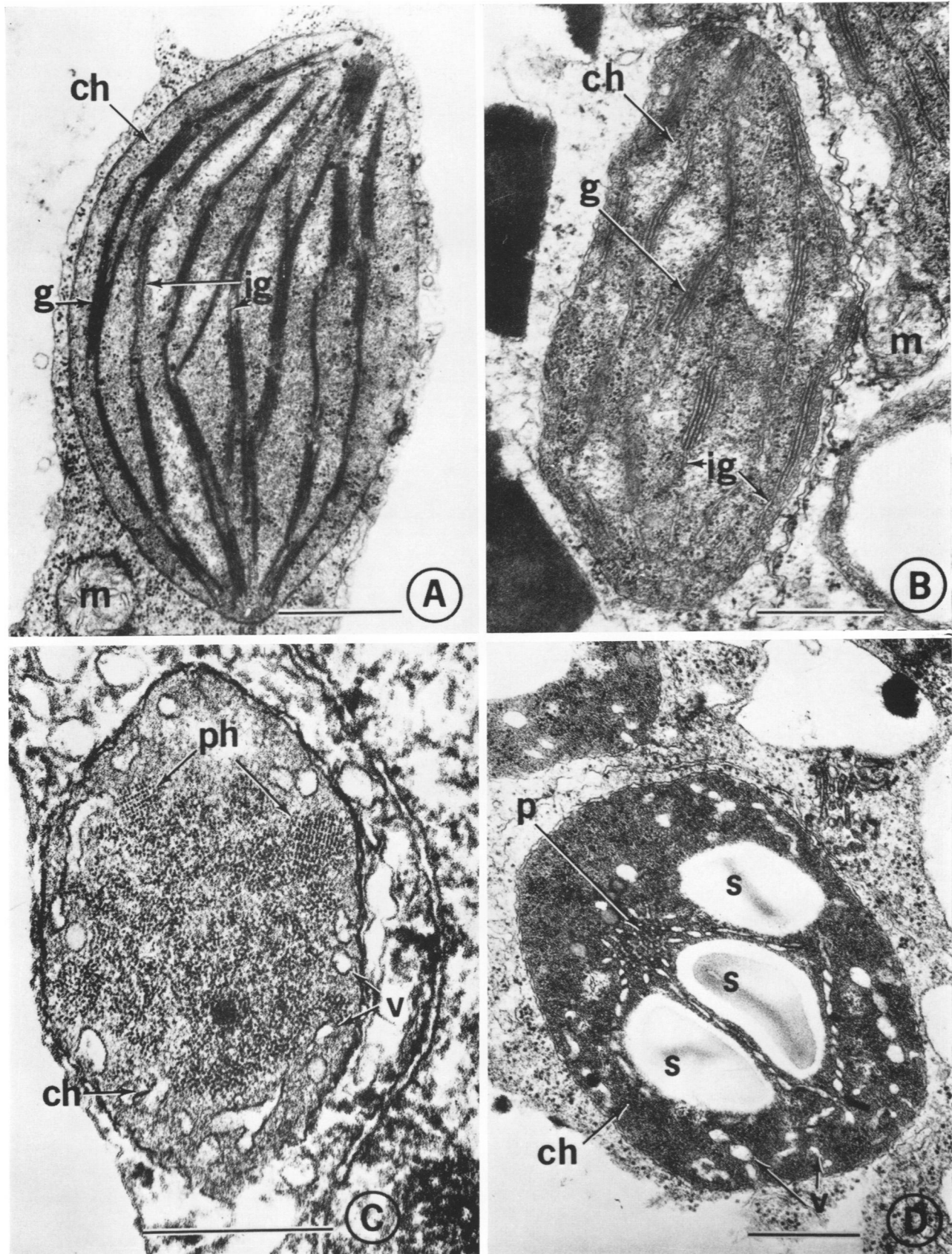
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Fig. 3. Differential response of plastids in cabbage cotyledons to light and tentoxin treatments. A: A normal chloroplast developed under continuous light. × 44,000. B: The same as part A except for toxin treatment during germination. No disorganization has occurred. × 44,000. C: A plastid containing phytoferritin in cotyledon tissue kept in darkness during germination. × 66,000. D: The same as part C except for toxin treatment during germination. Observe the prolamellar body with its vesicles and large starch granules. × 40,000. See Figure 2 for key.

prolamellar bodies were seen, but many plastids contained large amounts of a substance we assume to be phytoferritin. In the toxin-treated counterpart the reverse was true (Fig. 3D); nearly all plastids contained prolamellar bodies in the process of developing vesicles, but phytoferritin particles were seldom found. Starch was present, but the frequency of occurrence and the number of starch granules per plastid were lower than in the equivalent plastids from cucumber cotyledons.

In both toxin-treated and control cucumber and cabbage cotyledons, mitochondria appeared to be essentially normal (Figs. 2, 3), as were nuclei (not shown).

DISCUSSION

Tentoxin has no effect on the conversion of protochlorophyll-(ide) to chlorophyll(ide) or on the sigmoid shape of the time course plot of chlorophyll synthesis in cucumber seedlings, although it does cause decreased synthesis of these pigments and disruption of plastid structure. We conclude from these results that chlorosis induced in cucumber by tentoxin is not due to direct inhibition of chlorophyll synthesis. The slight stimulation of chlorophyll synthesis by the toxin in cabbage seedlings remains unexplained. No effects of the toxin were noted on mitochondria or nuclei at the ultrastructural level, but this does not preclude the possibility of effects on these organelles.

Impairment of starch degradation seems to be the most characteristic feature of toxin treatments, as revealed by electron microscopy, in both cucumber and cabbage, and it may be related to interference with granar development. These results demonstrate that cabbage is sensitive to the toxin, even though it does not show chlorosis.

We found that lamellar development was greatly reduced, but still observable in toxin-treated cucumber seedlings, and that etiolated, toxin-treated seedlings became uniformly pale green upon exposure to light. This suggests that chlorosis in cucumber seedlings is due to a reduction of chlorophyll synthesis within each chloroplast, rather than to nonuniform expression of toxicity among plastids.

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