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Adaptive Reorganization of Protein and Lipid Components in Chloroplast Membranes as Associated With Herbicide Binding

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Cultivation of Spirodela oligorrhiza (Kurtz) Hegelm on a sublethal dose of atrazine results in a higher linolenic to linoleic acid ratio in the thylakoid membrane lipids, less starch, more osmiophilic globules, and a reduced stroma lamellar system. Also, the grana become randomly oriented and contain more numerous and elongated lamellae. These alterations in the lipid composition and ultrastructure of the chloroplast resemble those previously observed in triazine-resistant weed biotypes and in chloroplasts developed under low light. Thylakoid membranes from atrazine-adapted plants revealed an additional high-affinity binding constant for [^{14}C]-diuron but the number of diuron binding sites actually decreased by 20 times compared to controls. The 32,000-dalton membrane protein of the chloroplast is synthesized actively, but its breakdown appears decreased compared to control plants. The adaptive reorganization of thylakoid components may be a compensatory mechanism for maintenance of a functional interaction of the proteins and lipids of the photosystem II complex.

Key words: Spirodela, thylakoids, atrazine, diuron, chloroplast, ultrastructure, 32,000-dalton protein

Adaptation of plants to different light intensities seems related to the ability of the chloroplast to organize the electron transport components for efficient photosynthesis [1–3]. Thus, plants grown at high light intensity differ from those grown at low intensity in structural, physiological, and biochemical characteristics, viz, lipid (fatty acids) quality of chloroplast thylakoids [4], ratio of chlorophyll to photosystem II reaction centers [2,5], arrangement of chloroplast membranes (thylakoid stacking) [1,6–8], and other biochemical parameters [9]. Differences in the ultrastructure and chemical composition of chloroplasts are well known between triazine-resistant and -susceptible plant biotypes [10,11]. Interestingly, higher and broader grana stacks and reduced stroma lamellae [8,12–15] in chloroplasts that develop under low light have

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also been observed in chloroplasts of plants treated with either diuron [12], bentazon [8,13], or atrazine [10,11,14,16], herbicides that inhibit electron transport at the donor site of photosystem II. Additionally, chloroplasts of plants treated with atrazine [14–16] have a higher unsaturation index of fatty acids for thylakoid lipids.

The mechanism that regulates the response of the chloroplast to light conditions and herbicide-adaptation is of considerable interest. The reoxidation of plastoquinone [1,4,19], the primary electron acceptor of photosystem II, is catalyzed by the B-protein. This reoxidation has been linked to the rates at which chloroplast electron transport is saturated by light. Triazines interfere with this reaction [17,18] by binding to a rapidly metabolized, 32,000-dalton thylakoid protein [20,21]. The rate of metabolism of this ubiquitous [22], surface-exposed membrane protein [23,24] is regulated by the intensity of light reaching the plant [25]. In vitro trypsinization of the thylakoids, or depletion of the protein in vivo, decreases electron transport at the reducing side of photosystem II [20]. Triazine-resistant plants normally synthesize [20,26] the 32,000-dalton protein but do not bind [^{14}C]-azidoatrazine [21]. Analysis of the decoded amino acid sequence of the gene for the 32,000-dalton protein from triazine-resistant and -susceptible Amaranthus hybridus biotypes has revealed a single amino acid difference [27]; however, whether this difference is the cause, or sole cause, of resistance to triazines is not known. A slightly altered conformation of this protein in the chloroplast membranes of some triazine resistant biotypes was revealed by in vitro trypsinization experiments [26]. Light and diuron also bring about conformational changes in the 32,000-dalton protein such that digestion by trypsin is altered [20]. Therefore, in addition to a change in its primary structure, microenvironment-related conformational changes may well affect the binding of atrazine to the protein in triazine resistant biotypes.

Spirodela oligorrhiza is an aquatic angiosperm well suited for studies on turnover of proteins and plastid development [28]. In this report we show that growth of Spirodela on nonlethal doses of atrazine brings about changes in the lipid quality and the ultrastructure of chloroplast membranes that resemble those seen in plants grown in low light and those observed in triazine-resistant biotypes. These findings are related to the turnover rate of the 32,000-dalton protein and altered binding of photosystem-II herbicides to thylakoid membranes.

MATERIALS AND METHODS

Analysis of Thylakoid Membranes

Spirodela oligorrhiza (Kurtz) Hegelm was cultured axenically for 15–20 days under steady-state light (1,500 lux, 25°C) in half-strength Hutner's mineral medium [29] supplemented with 0.5% sucrose in the absence or presence of 0.5 μM atrazine. Thylakoid membranes were isolated as previously reported [20]. Analysis of fatty acids, glycolipids, and phospholipids was carried out by the methods described earlier [10]. Herbicide binding with [^{14}C]-diuron ([3-(3,4-dichlorophenyl)-1,1-dimethylurea]; CIBA-GEIGY, Greensboro, NC) and [^{14}C]-atrazine ([2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine], CIBA-GEIGY) was carried out as described [30].

Microscopic Preparation

Spirodela fronds were sliced under 3% glutaraldehyde. Fixation, rinsing, and postfixation were carried out at 25°C in 0.05 M phosphate buffer, pH 6.8. Fixation

TABLE I. Fatty Acid Composition of Thylakoid Lipids From Control and Atrazine-Adapted Spirodela oligorrhiza

	Fatty acid composition (μmol/mg chlorophyll)					
Treatment	16:0	18:0	18:1	18:2	18:3	Total
Control	18.4 ± 0.4[a] (25.6)[b]	1.0 ± 0.0 (1.4)	1.4 ± 0.0 (1.9)	13.9 ± 0.5 (19.3)	37.3 ± 2.4 (51.8)	72.0 ± 3.5
Atrazine (0.5 μM)	23.9 ± 0.5 (26.8)	1.0 ± 0.0 (1.1)	1.7 ± 0.1 (1.9)	12.2 ± 0.2 (13.7)	50.4 ± 1.0 (56.5)	89.1 ± 1.4

[a]Mean values with associated standard deviations.
[b]Fatty acid composition expressed as mol percent of total thylakoid lipid (μmol/mg chlorophyll).

for 1.5 hr with 3% glutaraldehyde preceded washing with six changes of buffer over a period of 1 hr. The tissue was then postfixed in 2% osmium tetroxide for 2 hr, dehydrated in an acetone series, and embedded in Spurr's medium. Silver-gray sections were cut on a Sorvall MT-2 ultramicrotome with a diamond knife and mounted on uncoated copper grids of 300 × 75 mesh. The sections were stained with 2% uranyl acetate for 10 min, then with lead citrate for 5 min, and viewed in a Hitachi H-500* electron microscope. Structural analysis was based on randomly selected chloroplasts from cross sections of the fronds.

In Vivo Pulse Labeling

Plants were pulse-labeled with [^{35}S]-methionine (1,200 Ci/mmol; Amersham) for 3 hr in the mineral medium in which they were grown. In other treatments Spirodela plants were transferred to fresh mineral medium without sucrose and incubated overnight at 1,500-lux white light prior to labeling with [^{35}S]-methionine. In the chase experiments, the labeled plants were washed and incubated with the mineral medium containing 1 mM [^{32}S]-L-methionine. Samples were removed after 0, 1, 3, 7, 12, and 24 hr of chase. Then the plants were washed with distilled water and homogenized with 5 mM Tris-glycine (pH 8.5)-0.15 M NaCl. Soluble and membrane fractions were prepared [23] and fractionated on SDS 10–20% polyacrylamide gradient gels (SDS-PAGE) [22,25]. After fixing in 7% acetic acid-20% methanol, gels were prepared for fluorography (Autoflor, National Diagnostics) and exposed on curix RP1 X-ray films (Agfa).

RESULTS

Table I shows the fatty acid composition of thylakoid lipids of Spirodela cultured for 19 days in the absence or presence of 0.5 μM atrazine. Growth on atrazine resulted in a higher total thylakoid lipid and increased proportions of α-linolenic acid. Specifically, the fatty acid unsaturation, calculated as the ratio of 18:2 + 18:3/16:0, increased in monogalactosyldiglycerides and total phospholipids (Table II). These

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TABLE II. Lipid Unsaturation of Chloroplast Thylakoids

Biotype/treatment/reference	Lipid class[a]	Unsaturation ratio (18:2 + 18:3)/16
Spirodela oligorrhiza [this paper]	MGDG	
Control		0.80
0.5 μM Atrazine		1.42
Control	TPL	0.47
0.5 μM Atrazine		0.70
Lemna minor L [15]	MGDG	
Control		43.33
0.46 μM Atrazine		48.88
Senecio vulgaris L [10]	MGDG	
Sensitive		8.4
Resistant		13.7
Chenopodium album L [10]	MGDG	
Sensitive		18.5
Resistant		22.9
Amaranthus hybridus L [10]	MGDG	
Sensitive		8.9
Resistant		16.4
Brassica campestris L [11]	TPL	
Sensitive		1.94
Resistant		2.43

[a]MGDG = monogalactosyldiglyceride; TPL = total phospholipids.

lipid changes are qualitatively comparable to those reported for Lemna minor cultured on sublethal doses of atrazine [16] and for triazine-susceptible and -resistant weed biotypes [10,11] (Table II).

The changes in the lipid quality of the thylakoid membranes were coincident with reorganization in the chloroplast structure as revealed by electron microscopic observations. Chloroplasts from Spirodela plants grown under 2,000-lux white light exhibited fine structural features similar to those from other normally grown plants. The chloroplasts contained starch granules, a well-developed internal membrane system that consisted of stacked grana lamellae and interconnecting stroma lamellae, small osmiophilic bodies, and an electron-dense matrix [13] (Fig. 1). The grana, which averaged 400 μm in diameter, consisted of ten lamellae/granum (average maximum number). The stroma lamellae that interconnect the grana were 100–400 μm long (Table III).

When Spirodela plants were grown under 2,000-lux white light in a medium containing 0.5 μM atrazine, the number of lamellae/granum increased to 21, the average diameter of the grana increased to 870 μm, the length of the interconnecting stroma lamellae decreased to 75–100 μm, and the number of osmiophilic bodies doubled (Table III). In addition to these changes, the orientation of the grana became less regular, ie, the grana were more randomly situated (Fig. 2).

These altered features of the chloroplasts reverted to those of the control plants after transfer of atrazine-adapted plants to autotrophic mineral medium without the herbicide (Fig. 3). The length of the stroma lamellae increased to 100–400 μm (Table

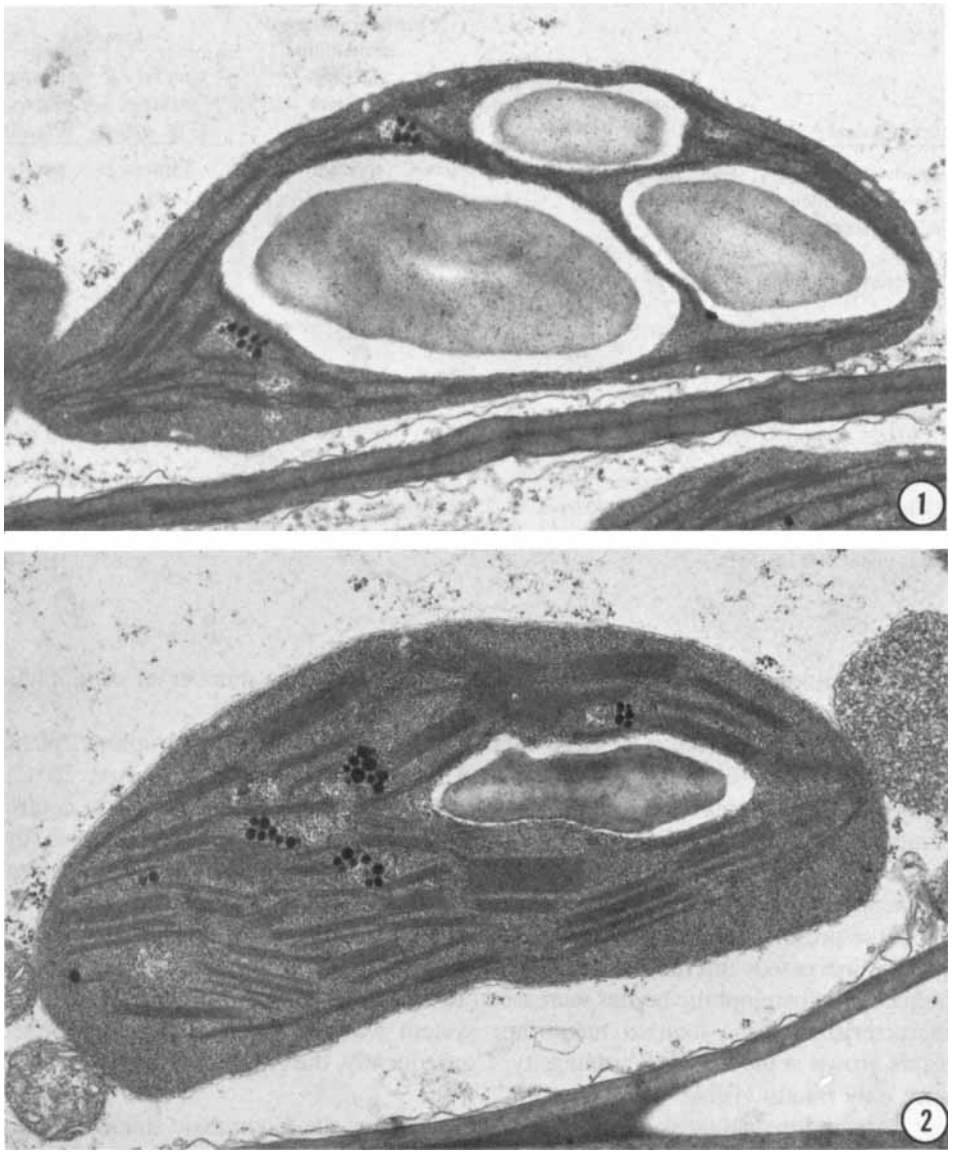


Fig. 1. Normal chloroplast from tissue grown at 2,000-lux white light. The chloroplast contains starch and a well-developed grana and stroma lamellae system. × 18,000.

Fig. 2. Chloroplast from tissue grown at 2,000-lux light in medium containing 0.5 μM atrazine. Stroma lamellae system is greatly reduced, whereas the grana lamellae are more elongated. × 21,000.

TABLE III. Summary of Data on Chloroplast Structures of Spirodela oligorrhiza*

Measurement	High light			Low light	
	Control plants (Fig. 1)	Atrazine-adapted (Fig. 2)	Atrazine-adapted plants transferred to medium lacking Atrazine (Fig. 3)	Control plants (Fig. 4)	Atrazine-adapted (Fig. 5)
Length of stroma lamellae that interconnect adjacent grana (μm)	100–400	75–100	100–400	150–400	50–100
Diameter of grana (μm)	400	870	470	430	870
Maximum No. grana lamellae/granum	10	21	15	11	17
No. osmiophilic bodies/chloroplast	9.7	22.9	10.8	11.3	18.2
No. starch bodies/chloroplast	2.2	0.24	1.6	2.8	2.6

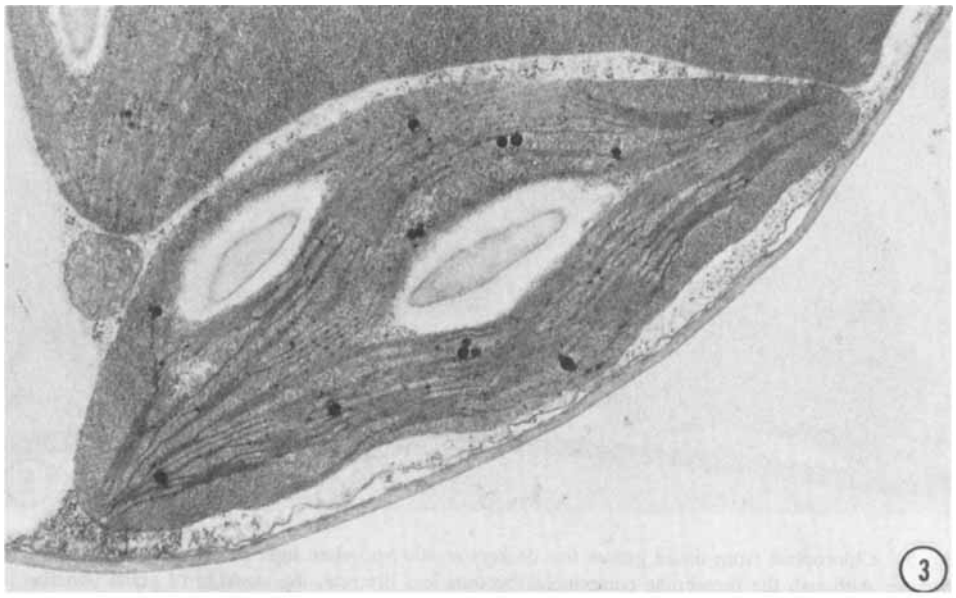
*Data based on average measurements from 10–25 chloroplasts. Chloroplasts were randomly selected from cross sections of the fronds. High-light plants were grown under 2,000-lux light and the low-light plants under 400-lux light.

III). In addition, the average diameter of the grana and the number of osmiophilic bodies decreased to 470 μm and 10.8, respectively.

To evaluate the effect of light intensity on the structure of chloroplasts, plants were grown with or without atrazine at a lower light intensity (400 lux, 25°C). Chloroplasts from the control plants without the herbicide resembled those of control plants grown at a higher light intensity (Fig. 4), while in the presence of atrazine, the chloroplasts from plants grown at low light intensity exhibited many of the features characteristic of plastids from plants grown with atrazine at high light intensity (Fig. 5). In the presence of atrazine, the diameter of the grana and the number of lamellae/granum increased, the lengths of the stroma lamellae were greatly reduced, and the frequency of osmiophilic bodies increased (Table III). In addition, the discrete staining characteristic of the internal membrane system was lost in the herbicide-adapted plants grown at the lower light intensity. Consequently, the grana and stroma lamellae were only faintly visible.

No major qualitative differences were found in the Coomassie-stained protein pattern between the normal plants and those adapted to atrazine except for the 26,000-dalton apoprotein of the light harvesting chlorophyll a/b complex, which stained more intensely in the atrazine-adapted plants (data not shown). This increase in quantity might be related to the enhanced formation of grana stacks (cf Fig. 2) [32,33].

In general, the SDS-PAGE patterns of the in vivo synthesized soluble (Fig. 6, lanes 3 and 4) and membrane (lanes 1 and 2) proteins were similar in control and atrazine-adapted plants pulse-labeled with [^{35}S]-methionine. Among the newly synthesized membrane proteins, the 32,000-dalton protein was a major product in both cases. However, in plants that were previously adapted to atrazine, transfer to



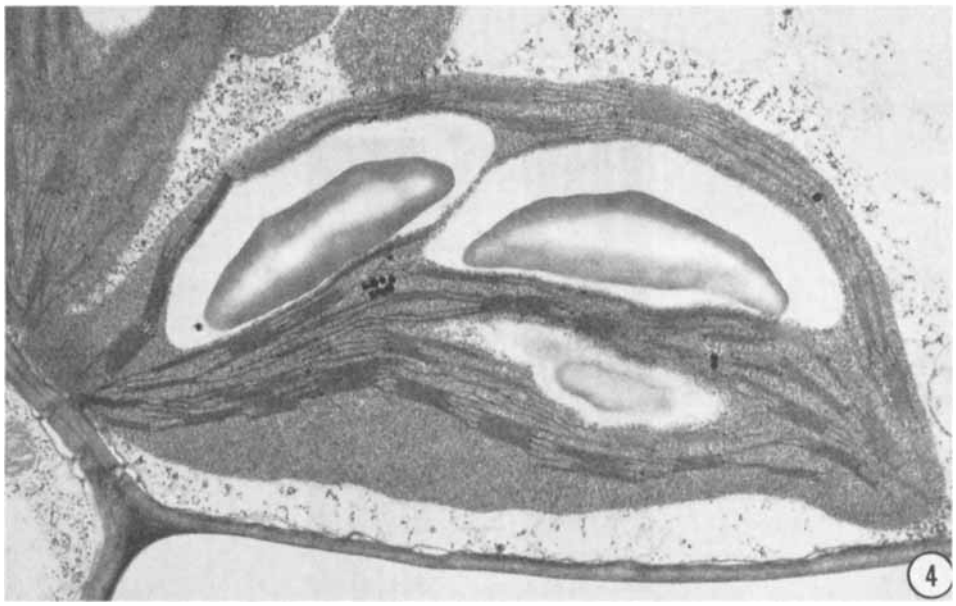


Fig. 3. Chloroplast from plants that were previously grown at 2,000-lux white light in a medium containing 0.5 μM atrazine for 19 days and then transferred to the mineral medium without the herbicide and incubated 14 hr. Chloroplasts exhibit starch and a normal complement of grana and stroma lamellae. × 22,000.

Fig. 4. Chloroplast from tissue grown for 19 days at 400-lux white light. The organelle tends to exhibit a structure similar to that found in plants under higher light intensity. × 20,000.

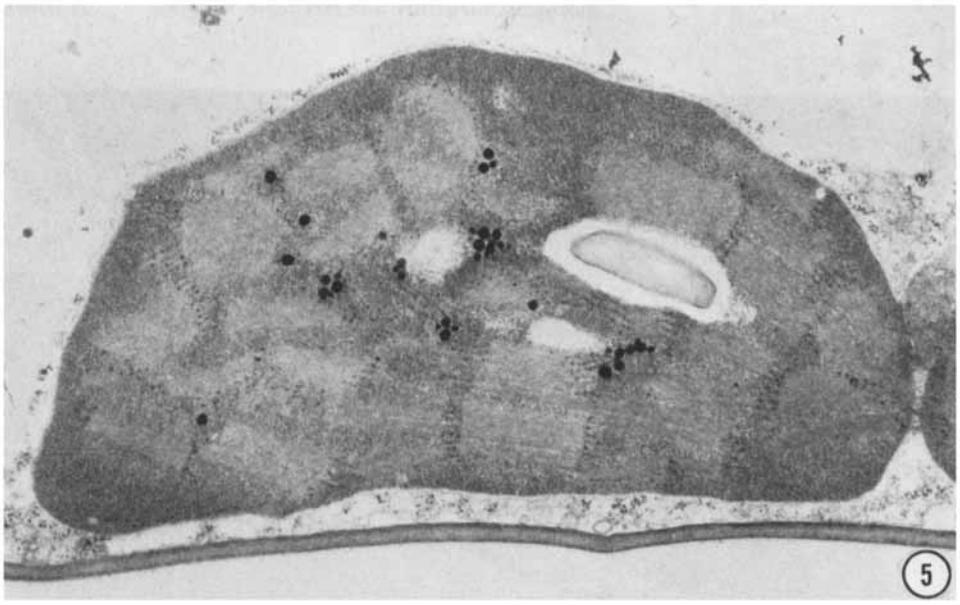


Fig. 5. Chloroplast from tissue grown for 19 days at 400-lux white light in the presence of 0.5 μM atrazine. Although the membrane components become less discrete, the stacking of grana lamellae is greatly increased but the length of the stroma lamellar system is reduced. × 20,000.

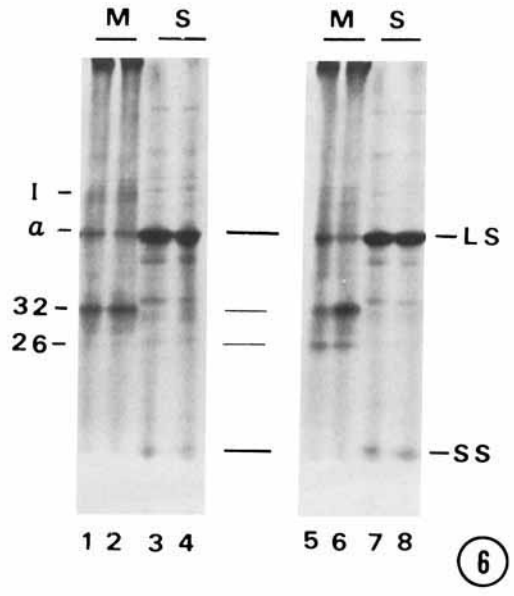


Fig. 6. SDS-PAGE patterns of [^{35}S]-methionine-labeled membrane-associated (M) and soluble (S) proteins of control (lanes 1 and 3) and atrazine-adapted (lanes 2 and 4) Spirodela. Steady-state, light-grown (1,500-lux white light) plants were radiolabeled for 3 hr with [^{35}S]-methionine, washed, and fractionated into soluble and membrane proteins. Lanes 5–8 are derived from 19-day-old control (lanes 5 and 7) and atrazine-adapted plants (lanes 6 and 8) pulse-labeled as before but after they were transferred for 14 hr to fresh, sucrose-free medium without the herbicide. Samples containing equal amounts of chlorophyll (M) or protein (S) are shown. The positions of I (a high M_r value subunit of the PSI reaction center), α (α-subunit of ATPase), LS and SS (large and small subunits of Rubisco), 32 (32,000-dalton protein), and 26 (apoprotein of the chlorophyll a/b light-harvesting complex) are indicated.

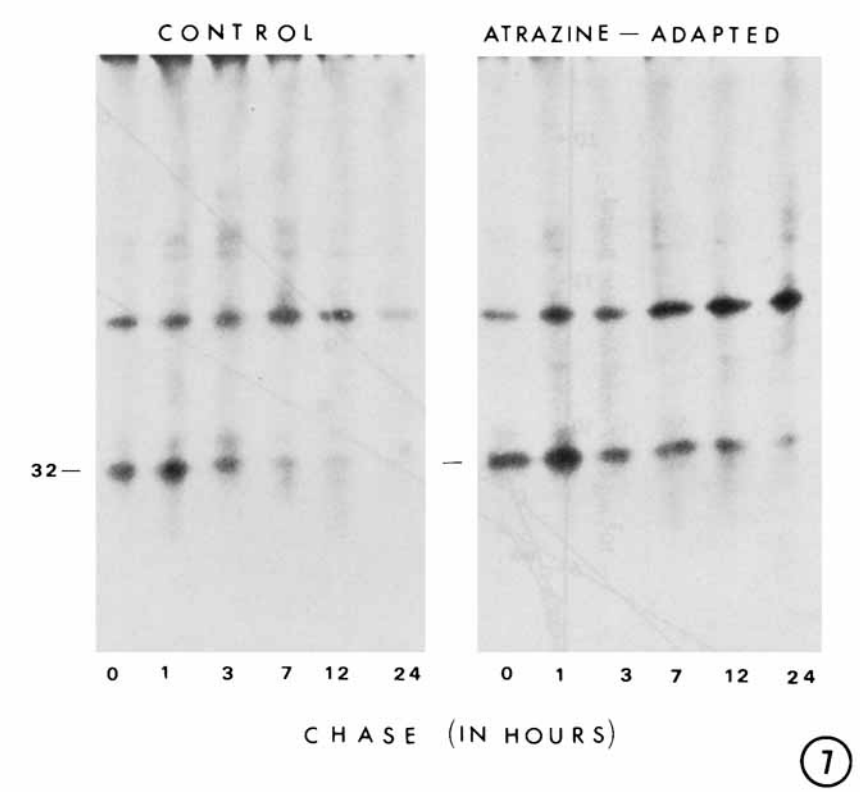


Fig. 7. The rate of in vivo breakdown of the 32,000-dalton protein in control and atrazine-adapted Spirodela. Steady-state, light-grown Spirodela plants were pulsed for 3 hr with 50 μCi/ml of [^{35}S]-methionine at 2,000 lux, then washed and incubated with culture medium containing 1 mM [^{32}S]-L-methionine. Samples were removed at the times of chase indicated. Cell membranes were isolated and analyzed by SDS-PAGE on an equal radioactivity basis.

atrazine-free medium resulted in an increase in the synthesis of the 32,000-dalton protein and a decrease in that of the α-subunit of the plastid ATPase relative to control Spirodela plants (Fig. 6, lanes 5 and 6), while the patterns of synthesis for soluble proteins were similar (lanes 7 and 8).

The rate of 32,000-dalton protein degradation was compared in control and long-term atrazine-adapted Spirodela. Pulse and chase experiments (Fig. 7) confirmed the previous results [25, 34, 35] on the rapid breakdown of the 32,000-dalton protein in normal plants. However, the breakdown of the 32,000-dalton protein in atrazine-adapted plants appeared retarded (Fig. 7). Rapid breakdown of the 32,000-dalton protein in these plants appeared to resume after their transfer to fresh, atrazine-free medium (not shown).

Differences between the control and atrazine-adapted Spirodela were also found in the ability of thylakoid membranes from these plants to bind [^{14}C]-atrazine and [^{14}C]-diuron. The double-reciprocal plots of herbicide binding were biphasic (Fig. 8), an observation recognized previously [36,37], and revealed significantly altered ability of atrazine-adapted plants to bind the two herbicides. The high-affinity atra-

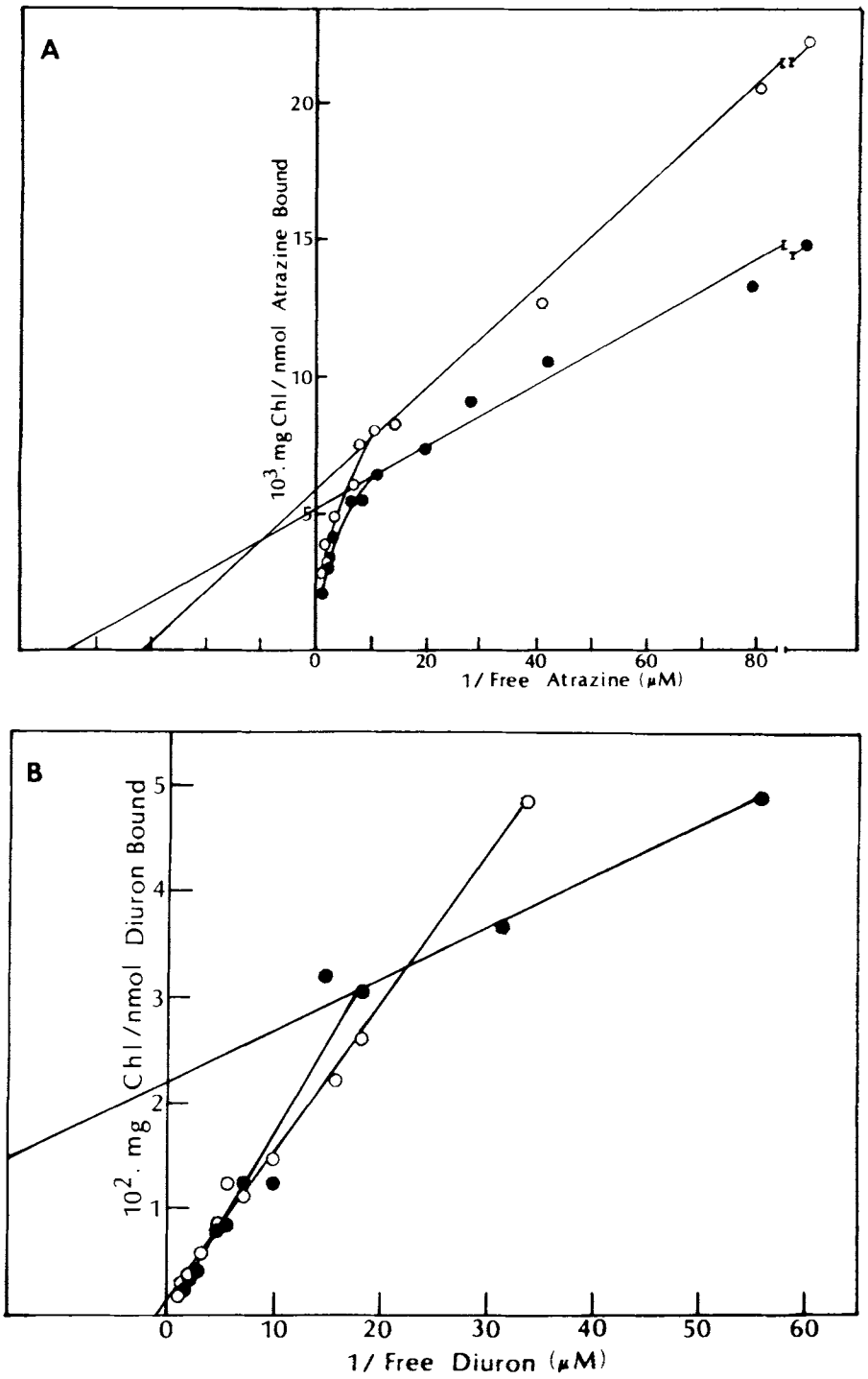


Fig. 8. Double-reciprocal plots of [^{14}C]-atrazine A) or [^{14}C]-diuron B) binding to thylakoid membranes isolated from control (○) and atrazine-adapted (●) Spirodela.

zine-binding constant (3.17×10^{-8} M) for Spirodela chloroplast membranes is comparable to binding constants for other thylakoid membranes [30, 36, 37]. The binding constant for atrazine in atrazine-adapted Spirodela membranes was slightly lower (2×10^{-8} M) with little change in the number of binding sites (Fig. 8A). However, the kinetics of binding diuron were markedly different for membranes from atrazine-adapted plants as compared to those from the control plants (Fig. 8B). The membrane from atrazine-adapted Spirodela revealed an additional high-affinity constant (2.17×10^{-8} M) for binding diuron compared to controls (1.33×10^{-6} M). However, only a few high-affinity binding sites were available in the thylakoid membranes from atrazine-adapted plants; the vertical intercept values indicated 20 times more [^{14}C]-diuron binding sites per mg chlorophyll in control membranes than in atrazine-adapted membranes.

DISCUSSION

Atrazine adaptation in Spirodela involves modifications in the quality of lipids and the ultrastructure of the chloroplast. These observations may be associated with an altered pattern of diuron binding to thylakoid membranes and an altered rate of metabolism for the 32,000-dalton thylakoid membrane protein. Thus, while synthesis of the 32,000-dalton protein is maintained in both herbicide-adapted Spirodela and triazine-resistant biotypes [26], the conformation of the protein in the membrane is apparently changed. Light, as well as diuron, causes conformational changes in this protein [20]. Moreover, the 32,000-dalton protein in the thylakoid membranes of some triazine resistant biotypes shows an altered susceptibility to trypsin digestion versus sensitive biotypes [26]. The lipid environment is known to affect lateral mobility and conformational flexibility of integral membrane proteins [38]. The Spirodela system described here provides a good model for studying lipid-protein interactions in the thylakoid membranes with respect to the metabolic flexibility of the 32,000-dalton protein.

The increase in glycerolipid in thylakoids from the triazine-adapted tissue is consistent with the observed differences in chloroplast structure. An increase in the diameter of the grana and number of thylakoids per grana stack (Table III) suggests that more lipid is needed to construct thylakoid membranes in the triazine-adaptive plant. A pattern in fatty acid composition characteristic of the individual tissues is also evident from these data. The lipids of chloroplast thylakoids from triazine-adapted tissues have a high proportion of polyunsaturated fatty acyl substituents, particularly linolenic acid. The increased proportions of linolenic acid paralleled an increased unsaturation level for the fatty acids of the monogalactosyldiglycerides and total phospholipids (Table II); these trends were qualitatively comparable to those reported for Lemna cultured on sublethal doses of atrazine [16] and for triazine-susceptible and -resistant weed biotypes [10,11].

Lipids with high proportions of polyunsaturated fatty acid constituents, particularly linolenic acid, have been assigned a functional role in maintaining membrane fluidity and in regulating the balance between lamellar and nonlamellar lipid structures in the chloroplast membrane [39]. Our data support this conclusion. Weed biotypes that are resistant to triazines and Spirodela and Lemna that were cultured on sublethal doses of triazines have more unsaturated thylakoid lipids. This observation may be pertinent to the hypothesis [40, 41] that the reaction of Q and B involves movement

of the reactants. According to this hypothesis the approachment of Q and B might be hindered by a loss of "a certain site" (triazine-binding site?) on the protein. A more unsaturated membrane would then compensate the defect of the protein and facilitate the reaction of the two (transfer of electrons from Q to B). However, the actual role of lipids in the latter reaction needs to be defined and is worthy of future experimentation. Conformational flexibility of the 32,000-dalton thylakoid protein could be relevant in this regard. In herbicide-resistant biotypes, in addition to a small change in the primary structure of the protein, specific alterations in the lipid environment of the protein may mediate conformation, orientation, and function of the protein in the photosystem II. The reorganization of thylakoid components may be a compensatory mechanism for maintenance of a functional interaction of the regulatory proteins, such as the 32,000-dalton protein, and lipids of the photosystem II complex. Such a mechanism could be activated in triazine-resistant biotypes, Spirodela, and Lemna cultured on nonlethal doses of atrazine, and in chloroplasts developed under minimum light.

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