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GLYPHOSINE, A PLANT GROWTH REGULATOR, AFFECTS CHLOROPLAST MEMBRANE PROTEINS

JANET P. SLOVIN and ELAINE M. TOBIN

Biology Department and Molecular Biology Institute, University of California, Los Angeles, CA 90024 (U.S.A.)

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Glyphosine (*N,N*-bis(phosphonomethyl)glycine) is known to increase sucrose levels in sugarcane and to cause chlorosis in maize and other plants. It has been suggested (Crofts, S.M., Arntzen, C.J., Vanderhoef, L.N. and Zettinger, C.S. (1974) Biochim. Biophys. Acta 335, 211–217) that its primary mode of action is to inhibit the synthesis of plastid rRNA. Growth of *Lemna gibba* L. G-3 on $5 \cdot 10^{-4}$ M glyphosine causes the plants to produce fronds lacking chlorophyll. The plastids in these white fronds contain only a few internal membrane structures, some of which are stacked. Sodium dodecyl sulfate polyacrylamide gel electrophoresis shows an accumulation of substantial amounts of both the large and small subunits of ribulosebisphosphate carboxylase by the white fronds. The membrane fraction from these fronds contains only traces of the light-harvesting chlorophyll *a/b* apoprotein in comparison to control plants. In vivo labeling and immunoprecipitation show that the large subunit of ribulosebisphosphate carboxylase is actively synthesized by the white fronds. However, labeling of the chlorophyll *a/b* apoprotein and a 32 000 dalton protein in the membrane fraction is extremely low compared to control plants. We conclude that in *Lemna*, glyphosine differentially affects the synthesis and/or processing of soluble proteins and some membrane chloroplast proteins, and could be useful in understanding the biogenesis of chloroplast membranes.

Introduction

The synthesis of chloroplasts involves a complex set of processes requiring synthesis of pigments, proteins and other membrane components and their assembly into a fully functioning structure. In order to understand the complete process, it is often useful to perturb the normal process so that intermediate steps can be studied. The results presented here suggest that glyphosine (*N,N*-bis(phosphonomethyl)-glycine) can be used in such a manner.

Glyphosine is a plant growth regulator which causes chlorosis in emerging leaves of several species of plants [1,2]. It is used commercially to increase sucrose levels in sugarcane by spraying plants shortly before harvest. In soybean seedlings, glyphosine was reported to inhibit growth, increase extractable phenylalanine ammonia-lyase activity, and decrease chlorophyll and anthocyanin content [3].

Croft et al. [1] found no effect of glyphosine on photophosphorylation or light-induced electron transport in isolated maize chloroplasts. They also studied its effect on intact maize seedlings, and reported that 5–6 days after foliar application, the number of chloroplast ribosomes and the level of chloroplast rRNA in the chlorotic segments were reduced. Glyphosine had no apparent effect on cytoplasmic ribosomes in the same tissue. On the basis of these data, Croft et al. [1] suggested that the primary mode of action of glyphosine is to inhibit the synthesis of plastid rRNA. The induced lack of plastid rRNA would then presumably lead to failure to synthesize chloroplast components and thus to chlorosis.

Abbreviations: SDS, sodium dodecyl sulfate; Chl, chlorophyll.

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There are a number of other reports correlating chlorosis or bleaching of photosynthetic pigments with the absence of chloroplast ribosomes in mutants [4–6] and in herbicide- [7–9] or heat-treated plants [10]. In some of these cases [4,5,7–9] the absence of ribosomes has been shown to be a secondary effect due to the sensitivity of chloroplast ribosomes to light in tissue lacking pigment.

When duckweed, *Lemna gibba* L. G-3, is grown on medium containing sucrose and 10^{-3}–10^{-4} M glyphosine, the plants continue to grow but new fronds are white (Jaworski, E., personal communication). We shall present evidence that glyphosine can cause chlorosis in *L. gibba* while the plastid ribosomes remain functional, and that it can differentially affect soluble and thylakoid membrane proteins. Thus, this compound could prove to be a useful tool for studying chloroplast membrane synthesis and assembly.

Materials and Methods

Materials. Glyphosine was obtained from Monsanto Corp. (St. Louis, MO) as Polaris, a powder of at least 99.9% purity. High specific activity [^{35}S]methionine (800–1400 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Proteins for molecular weight calibration of polyacrylamide gels were obtained from Sigma. Antibody to ribulosebisphosphate carboxylase from tobacco was a generous gift of Dr. S.G. Wildman, University of California, Los Angeles. Heat-killed, formalin-fixed *Staphylococcus aureus* was obtained as IgGsorb from The Enzyme Center, Inc. (Boston, MA).

Plant culture. L. gibba L. G-3 was grown aseptically on liquid E medium, which contains 1% sucrose in addition to salts and minor elements [11], at 28°C under constant light. Glyphosine was added to the medium before sterilization. Growth was measured by counting the number of fronds, visible without dissection, at various times after inoculation of 50 ml of medium with four green fronds. Spectra of intact fronds were obtained with an Aminco DW-2 spectrophotometer (American Instrument Co., Silver Springs, MD).

Electron microscopy. Tissue was fixed at room temperature in 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.0, rinsed with buffer three times, and post-fixed in 2% OsO_4 in the same buffer. Following three rinses with buffer, the tissue was dehydratec in an ethanol series, and embedded in Spurr's epoxy Fixation, rinsing and dehydration through the 30% ethanol step were performed in vacuo to remove aii trapped in the aerenchyma tissue. Thin sections were stained with lead citrate and uranyl acetate, and were photographed with a Phillips 200 electron microscope.

SDS-polyacrylamide gel electrophoresis. Electrophoresis was performed on a 12.5% polyacrylamide gel slab with a 4.75% polyacrylamide stacking gel as previously described [12]. Molecular weight calibration proteins (with molecular weights specified by the manufacturer) used were: bovine serum albumin, 66 000; ovalbumin, 45 000; trypsinogen, 24 000 β-lactoglobulin, 18 400; and cytochrome *c*, 12 500 They were co-electrophoresed with the samples. Gels were stained with Coomassie blue R 250. After destaining, gels were prepared for fluorography according to the method of Bonner and Laskey [13] and exposed to preflashed X-ray film (XR-5, Kodak) according to the method of Laskey and Mills [14].

Sample preparation. Two or three fronds were frozen in liquid nitrogen and ground in a glass homogenizer in 1 ml of ice-cold 10 mM Tris-HCl buffer (pH 7.4), 1.0 mM phenylmethylsulfonyl fluoride and 1.0% 2-mercaptoethanol. The soluble proteins were separated from a crude membrane preparation by a modification of the method of Cashmore [15]. After grinding, the extract was centrifuged for 12 s in a Beckman microcentrifuge to remove debris. The resulting supernatant was separated into soluble and insoluble components by centrifugation at 40 000 × *g* for 30 min. The majority of these proteins are thylakoid membrane proteins [15]. Soluble fractions were prepared for electrophoresis by precipitating the proteins in 90% (v/v) acetone and dissolving the precipitate in sample buffer (0.03 M Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 9% glycerol and 0.001% (w/v) bromophenol blue). Membrane fractions were dissolved in the same buffer. Samples were heated at 98°C for 2 min prior to loading on the gel.

In vivo labeling of proteins. To examine protein synthesis, two or three fronds were individually incubated on 9-μl drops of high specific activity [^{35}S]-methionine for 1 h, rinsed well with water, blotted dry, and frozen in liquid nitrogen. Glyphosine (10^{-3} M) was included for the incubation of glyphosine-treated plants. They were then either stored

at −80°C or ground immediately as described above.

Immunoprecipitation. Immunoprecipitates of ribulosebisphosphate carboxylase were obtained with specific antibodies to the tobacco holoenzyme [16], according to the method of Kessler [17] as modified by Ivarie and Jones [18]. Aliquots (35–50 μl) of the radioactively labeled soluble fraction were preincubated with freshly washed IgGsorb to remove radioactivity which might bind nonspecifically to the *Staphylococcus* cells. The cells were removed by centrifugation, and the supernatant was incubated with 15 μl of the antiserum for 20–30 min in buffer A: 50 mM Tris-HCl (pH 7.6), 0.1 M NaCl, 0.1% Triton X-100, at room temperature. Titration of the soluble fraction from fully green plants with varying amounts of antiserum showed that at this ratio of antiserum to homogenate the antibody is in excess. The antibody-antigen complex was precipitated by addition of 120 μl of a 10% suspension of freshly washed IgGsorb, washed with buffer A, and dissociated from the *Staphylococcus* cells by boiling for 2 min in buffer A containing 2.0% SDS and 6 M urea. Bromophenol blue and glycerol were added to the sample prior to electrophoresis.

Results

L. gibba can be grown in the light in the presence of $5 \cdot 10^{-4}$ M glyphosine and 1% sucrose. Plants did not grow in 10^{-2} M glyphosine, and no effects on growth were visible at 10^{-5} M glyphosine. Green fronds placed on the glyphosine-containing medium remain green, but all new fronds produced during the first 3 weeks in culture are white or pale yellow. White fronds from glyphosine-treated plants rapidly become pale green and produce fully green offspring when placed on medium without glyphosine. Absorption spectra of intact fronds demonstrate that a white frond contains less than 0.4% of the chlorophyll of a green frond. Thus, growth in the presence of glyphosine is presumably not dependent on photosynthesis, but on the sucrose in the medium.

The plants growing on the glyphosine medium produce nearly as many new fronds as control plants during the first 3 weeks in culture (Fig. 1). New fronds are smaller and less gibbous than those of control plants.

Electron micrographs of chloroplasts in fronds

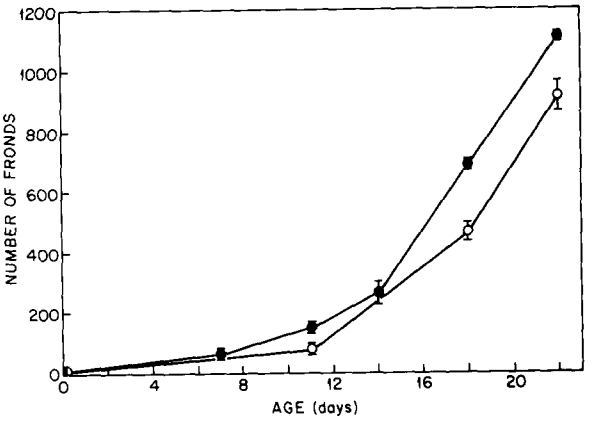
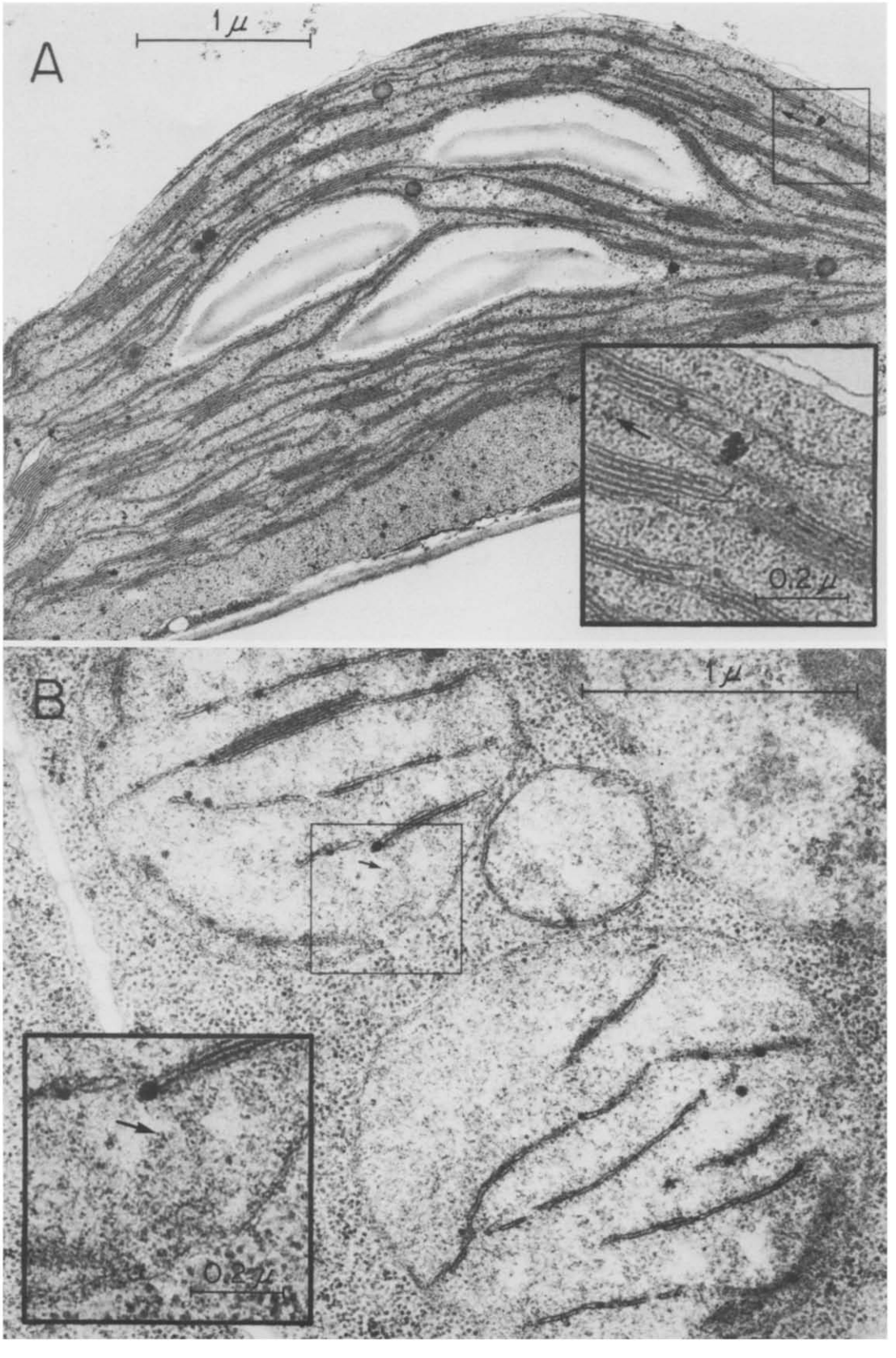


Fig. 1. Effect of glyphosine on growth of *Lemna*. Fronds were grown in E medium with or without $5 \cdot 10^{-4}$ M glyphosine. Growth was measured as the number of fronds in each of three flasks harvested on the days indicated. Plotted points are mean values ± S.D. •, E medium; ○, E medium + $5 \cdot 10^{-4}$ M glyphosine.

grown in the presence of glyphosine show that they contain many fewer thylakoid structures than normal tissue (Fig. 2). Those present appear to be capable of limited stacking. Ribosomes are clearly visible in both the cytoplasm and chloroplasts of both treated and untreated plants (arrows in Fig. 2).

The water-soluble and membrane-bound polypeptides present in normal and glyphosine-grown fronds are shown, after electrophoresis and staining, in Fig. 3. The majority of the membrane polypeptides have been shown to correspond to chloroplast thylakoid components [15]. Comparison of thylakoid membranes isolated by a flotation method [19] with the crude membrane fraction shown here confirm that result in *Lemna* (data not shown). This correspondence also remains valid for glyphosine-treated plants, although the total amount of thylakoid proteins per frond is less. Each lane shown in Fig. 3 contains proteins extracted from samples of equal fresh weight. The soluble proteins (S) show few differences between treated (+) and control (−) plants. Although the amount of soluble protein per mg fresh weight is less in the glyphosine-treated than in the control plants, the two subunits of ribulosebisphosphate carboxylase (marked by arrows) are clearly visible in both samples. In the membrane fraction (M), a doublet, which may correspond to the α and β sub-


A
1 μ
0.2 μ
B
1 μ
0.2 μ

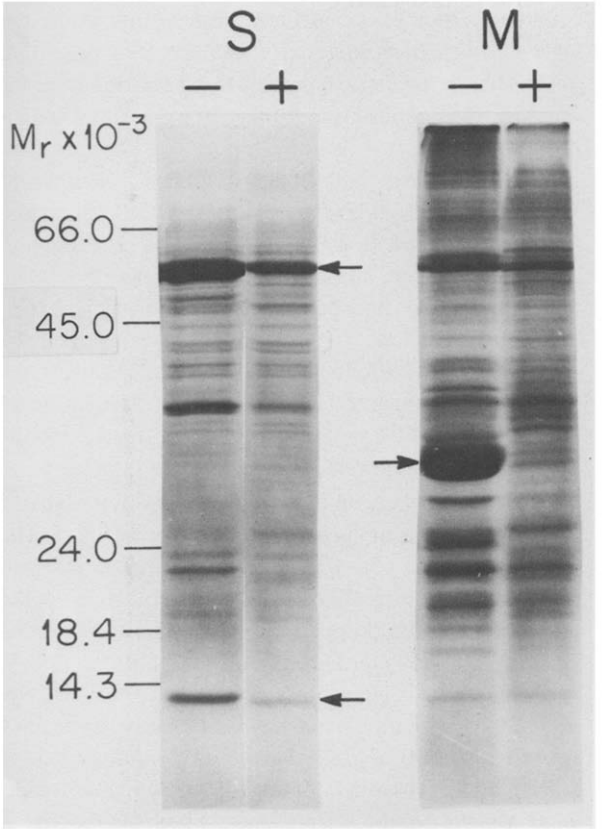


Fig. 3. Comparison of proteins contained in normal and glyphosine-treated plants. Soluble (S) and membrane (M) fractions were prepared as described in Materials and Methods from fronds grown without (−) or with (+) $5 \cdot 10^{-4}$ M glyphosine. After electrophoresis the gels were stained with Coomassie blue. The positions of the molecular weight standards are shown. Arrows indicate the position of the subunits of ribulosebisphosphate carboxylase in the soluble fraction and of the Chl *a/b* apoprotein in the membrane fraction.

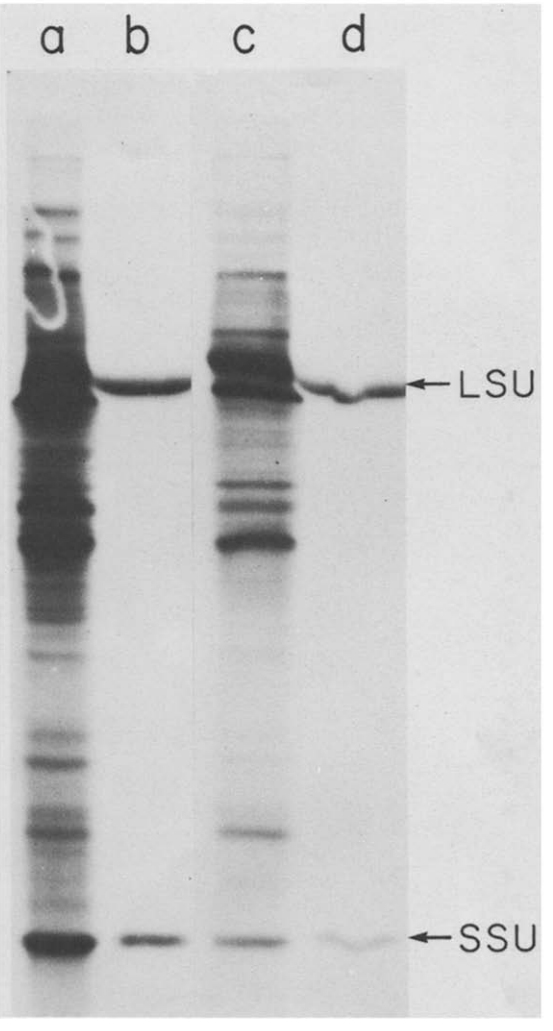


Fig. 4. Synthesis of soluble proteins in normal and glyphosine-treated plants. Fluorogram of soluble proteins and immunoprecipitated ribulosebisphosphate carboxylase labeled in vivo with [^{35}S]methionine after electrophoresis on a 12.5% SDS-polyacrylamide gel (see Materials and Methods). The amounts loaded in each lane represent the amounts present in three fronds. The ratio of the fresh weights of these normal and glyphosine-treated fronds is approx. 2 : 1. The arrows indicate the large (LSU) and small (SSU) subunits of ribulosebisphosphate carboxylase. (a) Normal plants, (b) immunoprecipitate from a, (c) glyphosine-treated plants, (d) immunoprecipitate from c.

units of coupling factor, which are synthesized in the chloroplast [20], is present (at about 56 000 daltons) in both types of fronds. However, a number of other bands are greatly reduced or completely missing and some bands appear to be present in glyphosine-treated but not in normal green fronds. The most pro-

Fig. 2. Electron micrographs of chloroplasts in plants grown without (A) and with (B) $5 \cdot 10^{-4}$ M glyphosine in the medium. Fronds used for B were the bleached primary or secondary offspring of the initial green fronds placed in culture. Tissue was prepared for microscopy as described in Materials and Methods.

nounced difference is seen in the Chl *a/b* apoprotein band at about 28000 daltons [21]. This band, marked by an arrow in Fig. 3, is the major stained band in the normal green tissue (–) and is dramatically reduced in intensity in the treated tissue (+). Substantial differences can also be seen in polypeptide bands at about 24000, 27000 and in the range 33000–40000 daltons.

We examined the proteins being actively synthesized in vivo in normal and in glyphosine-treated plants (Fig. 4). Glyphosine does not inhibit the uptake of [^{35}S]methionine from the medium. The white fronds continue to synthesize most of the soluble proteins seen in the normal plants. Although the amount of radioactivity incorporated per frond into soluble proteins is less in the glyphosine-treated plants, the amount incorporated on a fresh weight basis is similar.

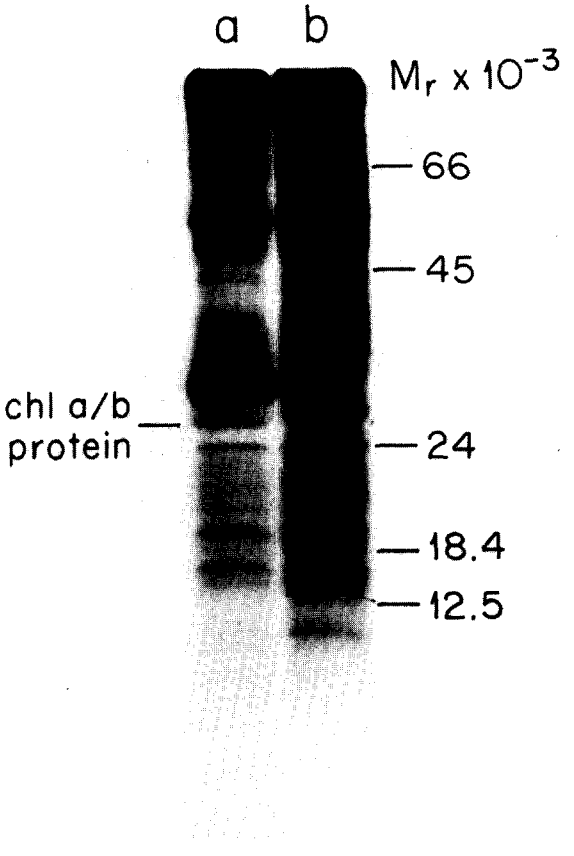


Fig. 5. Synthesis of membrane proteins in normal and glyphosine-treated plants. Fluorogram of membrane proteins labeled in vivo with [^{35}S]methionine after electrophoresis on a 12.5% SDS-polyacrylamide gel. Approximately equal counts, representing approximately equal fresh weights, were loaded on each lane. (a) Normal plants, (b) glyphosine-treated plants.

Both the large and small subunits of ribulosebisphosphate carboxylase are synthesized in treated and untreated plants (Fig. 4). After 1 h of labeling with [^{35}S]methionine, these subunits (LSU and SSU) can be seen in the fluorogram of total soluble proteins (lanes a and c). Their identity is confirmed by immunoprecipitation with antiserum against the tobacco holoenzyme (lanes b and d). The immunoprecipitate bands of the LSU are distorted by the presence of large amounts of IgG in the sample.

The fluorogram of the membrane proteins labeled with [^{35}S]methionine in vivo is shown in Fig. 5. The position of the Chl *a/b* apoprotein band was determined by staining [21,22] and is indicated on the fluorogram. This polypeptide is labeled in the normal green plants (Fig. 5, lane a) but not at all in the glyphosine-treated plants (lane b). The radioactivity associated with this band has been shown to be associated with the Chl *a/b* apoprotein by immunoprecipitation [21]. There is a band of slightly higher molecular weight in the glyphosine-treated plants that is not seen in the fluorogram of the control sample.

It can also be seen (Fig. 5) that glyphosine-treated plants apparently do not synthesize the polypeptide at about 32000 daltons which is the most highly labeled polypeptide in the control plants. Other less noticeable differences can be seen on careful comparison of the two membrane samples.

Discussion

Glyphosine has been previously shown to decrease the number of chloroplast ribosomes and the amount of chloroplast rRNA in maize seedlings observed 5–6 days after treatment [1]. Croft et al. [1] suggest that the primary mode of action of glyphosine is to inhibit the synthesis of plastid rRNA. We have found that in *L. gibba*, glyphosine may cause chlorosis by another mechanism. The new *Lemna* fronds formed in the presence of glyphosine lack chlorophyll, but plastids which contain ribosomes and some partially stacked thylakoid membranes are present (Fig. 2). We have demonstrated that these plastid ribosomes are func-

tional. Electrophoresis of the soluble proteins of the bleached fronds reveals that they contain large amounts of the large subunit of ribulosebisphosphate carboxylase (Fig. 3), which is coded for by chloroplast DNA [23] and is translated on chloroplast ribosomes [24]. Studies of in vivo protein synthesis on the bleached fronds demonstrate active synthesis of the large subunit 3–4 days after glyphosine treatment. Therefore, the chloroplast ribosomes present in the white glyphosine-treated tissue can function to synthesize protein.

The plastid ribosomes seen in the electron micrograph (Fig. 2) may be ribosomes synthesized prior to glyphosine treatment. The available data are consistent with either inhibition of plastid rRNA synthesis or with eventual photodestruction of plastid ribosomes [4,5,7–9]. While it may be that glyphosine also acts to inhibit plastid rRNA synthesis in *Lemna*, our results suggest that the chlorosis is not primarily caused by failure of the plastid ribosomes to function.

We cannot tell from the experiments described what the primary action of glyphosine is. However, the differential effects of glyphosine on the synthesis of soluble proteins and some membrane proteins of chloroplasts make it an interesting experimental probe. While glyphosine had apparently little effect on the synthesis and accumulation of water-soluble proteins, substantial differences were observed between the proteins present in the membrane fractions of normal and treated plants (Fig. 3). The most dramatic of these was in the amount of the Chl *a/b* apoprotein.

In vivo labeling revealed that in fact the Chl *a/b* apoprotein is apparently not being synthesized in glyphosine-treated plants (Fig. 5). Along with the apparent decrease in synthesis of the Chl *a/b* apoprotein, there is also a substantial decrease in labeling of a polypeptide having a molecular weight of about 32 000 (Fig. 5). This polypeptide is the most heavily labeled band in the control plant sample and is most probably the rapidly synthesized and rapidly turned-over, herbicide-binding protein (P32000) described by Ellis [25], Reisfeld et al. [26] and Pfister et al. [27]. Unlike the Chl *a/b* apoprotein, P32000 is coded for by chloroplast DNA and is synthesized on chloroplast ribosomes [25]. In order to affect synthesis of both of these proteins, as well as several other membrane proteins, as seen in Fig. 5, glyphosine would have to act on cytoplasmic as well as chloroplastic synthesis of chloroplast membrane proteins.

Failure to synthesize, process, or insert one membrane component could affect the synthesis or insertion into the membrane of another component. For example, the apparent lack of synthesis of the Chl *a/b* apoprotein could be a consequence of abnormal processing if a failure to properly insert the protein into the membrane resulted in greatly increased rates of degradation of these polypeptides. Evidence for rapid turnover of newly synthesized Chl *a/b* apoprotein in a barley mutant which lacks chlorophyll has been presented [28].

The Chl *a/b* apoproteins in *Lemna* and other plants are synthesized as higher molecular weight precursor molecules [26,29], which must be processed and combine with chlorophyll in order to function normally as an integral part of the thylakoid membrane. Other chloroplast proteins, such as the small subunit of ribulosebisphosphate carboxylase (for a review see Ref. 29), are also synthesized as precursors. In the electrophoretic pattern of membrane proteins from glyphosine-treated plants (Fig. 3), a number of bands appear to be missing or shifted to a slightly higher molecular weight. The fluorogram of proteins being synthesized in vivo (Fig. 5) shows a new band in the sample from glyphosine-treated plants at a slightly higher molecular weight than the band labeled Chl *a/b* protein in the control sample. This figure suggests that glyphosine may affect the normal processing and/or insertion of this thylakoid membrane polypeptide either directly or indirectly.

Whatever the primary mode of action, we feel that this compound could provide a valuable tool for understanding the processes involved in synthesizing and assembling chloroplast membranes.

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