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# Light regulation of the synthesis of the large subunit of ribulose-1,5-bisphosphate carboxylase in peas: Evidence for translational control

**(chloroplast gene expression)**

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**ABSTRACT** **The specific activity of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) increases 30- to 50-fold when dark-grown pea seedlings are shifted into the light. The large subunit (LS) of this multimeric protein is known to be synthesized in the chloroplast, but plastids from dark-grown cells contain relatively low levels of LS. However, despite the low level of LS synthesis in the plastids of dark-grown plants, these organelles contain significant levels of LS mRNA. Hybridization studies showed that the amount of LS mRNA increased about 3-fold, relative to total plant RNA, when dark-grown plants were illuminated. This increase in LS mRNA can be accounted for by a similar increase in chloroplast genome copy number. It was found that the amount of translatable LS mRNA per μg of plastid RNA is similar when isolated from either dark-grown plants or dark-grown plants subjected to light. These results suggest that although light can increase the level of LS mRNA by increasing the copy number of this gene, the primary regulation of LS synthesis by light in pea chloroplasts is at the level of translation.**

Ribulose-1,5-bisphosphate carboxylase [Rbu$P_2$Case; 3-phospho-D-glycerate carboxylase (dimerizing), EC 4.1.1.39], the enzyme responsible for $CO_2$ fixation in photosynthesis, is a multimeric protein comprised of eight large subunits (LS) and eight small subunits (SS) (1). The enzyme is found in the chloroplast, but the chloroplast genome encodes only the LS; the genetic information for the SS is nuclear-coded. The SS is synthesized in the cytoplasm as a precursor, which is processed and transported into the chloroplast where it combines with the LS to form the active enzyme (2–5). Light is known to be required for the maturation of chloroplasts and to influence the expression of a large number of chloroplast and nuclear genes. Rbu$P_2$Case is one of the many plant proteins whose synthesis increases in the presence of light, but the effect of light on Rbu$P_2$Case synthesis varies considerably among species. Pea is an especially good plant in which to study the effect of light on the induction of Rbu$P_2$Case synthesis, because the level of this enzyme increases manyfold after exposure to light (6, 7).

Previous studies using pea seedlings have shown that the mRNA coding for the SS of Rbu$P_2$Case increases dramatically during exposure of the plants to light (7–9). This appears to be due to a specific increase in transcription, since the copy number of the SS gene in the nucleus was not significantly affected by light. The LS mRNA also increases after exposure of pea plants to light, but in this case there appeared to be a correlation between the rate of increase in LS mRNA and an increase in chloroplast genome copy number (8, 10). In the present studies, we have examined the effect of light on LS synthesis in pea. Our results show that light causes an increase in LS mRNA that is related to chloroplast genome copy number, and additional evidence is presented that light regulates LS synthesis at the level of translation.

## MATERIALS AND METHODS

**Plants.** Pea seeds (*Pisum sativum* var. Progress No. 9, Agway, Syracuse, NY) were sown in coarse vermiculite and grown in the light or dark at 25°C for various times. The buds were separated from the rest of the plant and immediately placed on ice prior to homogenization.

**Rbu$P_2$Case Assay.** Cell-free extracts were prepared by homogenizing the pea buds in an Omnimixer (DuPont) with a buffer (10 ml/g) containing 100 mM Tris Cl (pH 8.0), 20 mM $MgCl_2$, 10 mM dithiothreitol, and 1% (wt/vol) polyvinylpyrrolidone. The homogenate was filtered through Miracloth (Calbiochem) and the filtrate was centrifuged at 10,000 × *g* for 10 min. An aliquot of the resultant supernatant was preincubated at 30°C for 10 min in a buffer that contained, in a final volume of 50 μl, 40 mM Tris Cl (pH 8.0), 16 mM $MgCl_2$, 3.6 mM dithiothreitol, 0.8 mM EDTA, and 10 mM $NaH^{14}CO_3$ (5,000 cpm/nmol). The Rbu$P_2$Case reaction was initiated by the addition of ribulose 1,5-bisphosphate at a final concentration of 1.5 mM. After 10 min at 30°C, 50 μl of 1 M HCl was added to terminate the reaction. The reaction mixtures were transferred to scintillation vials and evaporated to dryness to liberate free $^{14}CO_2$. Each dried sample was dissolved in 0.1 ml of water and the radioactivity was measured in a liquid scintillation spectrometer.

Protein concentrations were determined by the method of Lowry *et al.* (11), with bovine serum albumin as the standard.

**Preparation of Plastids and Plastid RNA.** Plastids were isolated from pea seedlings by the procedures of Chua and Schmidt (5) and Morgenthaler *et al.* (12) as modified by Fish and Jagendorf (13). Tissue was homogenized briefly in a buffer (5 ml/g) containing 50 mM Hepes/KOH (pH 8.5), 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM ascorbic acid, and 3 mM EGTA (buffer A). The homogenate was filtered through two layers of Miracloth and the filtrate was centrifuged for 3 min at 5000 × *g* in a GSA rotor (Sorvall). A pellet from 10–20 g of tissue was resuspended to a final volume of 3 ml in buffer A and layered onto a 30-ml linear gradient, consisting of 10–80% Percoll, 0.3–2.4% (wt/vol) polyethylene glycol 3350, and 0.1–0.8% (wt/vol) of both Ficoll 400 and bovine serum albumin, in buffer A containing 5.2 mM glutathione. The gradients were centrifuged for 7 min at 8000 rpm (Sorvall, HB-4 rotor) and the lower of the two bands in the gradient, which contained the intact plastids, was removed, diluted in a buffer containing 50 mM Hepes, (pH 8.4) and 330 mM sorbitol, and centrifuged. The isolated

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Abbreviations: Rbu$P_2$Case, ribulose-1,5-bisphosphate carboxylase; LS, large subunit(s); SS, small subunit(s); cpDNA, chloroplast DNA.

plastids were lysed by suspending the pellets in a buffer containing 40 mM Tris Cl (pH 7.4), 10 mM EDTA, proteinase K (200 μg/ml), and 2% (wt/vol) sodium sarkosyl and incubating the suspension at room temperature for 15 min. The lysate was extracted twice with equal volumes of a phenol/chloroform mixture (1:1, vol/vol) saturated with 10 mM TrisCl, pH 8.0/1 mM EDTA. Nucleic acids were precipitated from the aqueous phase by the addition of 0.1 vol of 3.0 M sodium acetate (pH 5.0) and 2.5 vol of absolute ethanol followed by an overnight incubation at −20°C. The precipitate was dissolved in water and the RNA was freed of DNA by precipitation with 3 M sodium acetate (14). The RNA was found to contain <3% contaminating DNA.

**Plastid Genome Quantitation.** Pea plants that had been grown either in the dark for 14 days or for 14 days in the dark followed by 72 hr of light were cut about 1.25 cm from the leaves. The stems then were immersed in 200 μl of water containing [$^3$H]thymidine (75 Ci/mmol, 1 mCi/ml; 1 Ci = 37 GBq) and incubated in either the light or dark for 3 hr at 25°C. The leaf tissue was then homogenized, and the nucleic acids were obtained as described above. The DNA was isolated as described by Murray and Thompson (15), by precipitation with cetyltrimethylammonium bromide. The DNA was purified further by chromatography on an Elutip-d column (Schleicher & Schuell) as described by the supplier, was quantitated by a fluorometric assay (16), and was subjected to alkaline hydrolysis to degrade RNA before being used for hybridization as described below.

The isolation of plasmid DNA containing restriction fragments of spinach chloroplast DNA (cpDNA) was carried out as described by Heinhorst *et al.* (17). Plasmids containing *Pst* I fragments of spinach cpDNA representing 72% of the large single-copy region and 15% of the inverted regions (18) were obtained from J. D. Palmer (University of Michigan). The plasmids were digested with *Pst* I and, after purification by electrophoresis, the fragments were mixed in equimolar amounts, denatured, and fixed to nitrocellulose membranes. The membranes were prehybridized at 37°C for 4 hr in 2 ml of a solution containing 50% formamide, 3× NaCl/Cit (1× is 0.15 M NaCl/15 mM sodium citrate, pH 7), 1× Denhardt's solution (19), 0.1% $NaDodSO_4$, and 1 mM EDTA. Hybridization to [$^3$H]thymidine-labeled DNA isolated from light- and dark-grown plants was carried out at 37°C for 44 hr in 1 ml of the above solution containing 50 μg of denatured calf thymus DNA. The nitrocellulose filters were rinsed and dried, and the radioactivity was measured in a liquid scintillation spectrometer. The values were corrected for the efficiency of hybridization (about 70%) of $^{32}$P-labeled *Pst* I fragments of spinach cpDNA to identical unlabeled DNA sequences that had been bound to a filter.

**RNA Dot Blot Hybridization.** RNA was isolated from dark- and light-grown plants as detailed above and hybridization was carried out as described (20). The RNA was applied to nitrocellulose sheets by use of a 96-hole Minifold apparatus (Schleicher & Schuell), baked for 2 hr at 80°C under reduced pressure, and then prehybridized at 42°C for 18–20 hr in a solution containing 5× NaCl/Cit, 50% formamide, 50 mM sodium phosphate (pH 6.5), 1× Denhardt's solution, and denatured salmon sperm DNA (100 μg/ml). For these experiments, a 0.7-kilobase (kb) DNA probe containing pea LS sequence was used. This probe was prepared from plasmid pPS10, which has a 3.7-kb fragment containing the pea LS gene. Cleavage of this plasmid with *Pst* I and *Xba* I yielded a 0.7-kb fragment that was isolated and then labeled by the random primer-extension method using [$\alpha^{32}$P]dNTPs (21). Isolation of pPS10 was facilitated by the availability of a plasmid containing a fragment of the LS gene (kindly supplied by J. D. Palmer). The labeled probe was hybridized to the RNA for 24 hr at 42°C. After washing, the blots were exposed to x-ray film at −70°C, and then the hybridized spots were excised, dissolved in Ready-Solv (Beckman), and assayed by liquid scintillation counting.

***In Vitro* Protein Synthesis.** The preparation and characteristics of the partially defined *in vitro Escherichia coli* system used have been described (22, 23), except that RNA polymerase was omitted from the incubations. The translations were carried out for 60 min at 37°C, and the amount of [$^{35}$S]methionine incorporated into protein was measured by spotting aliquots of the incubation onto glass fiber filters. The filters were heated at 100°C for 10 min in 10% $Cl_3CCOOH$, washed in 10% $Cl_3CCOOH$ followed by 100% ethanol, and assayed for radioactivity in a liquid scintillation spectrometer. The amount of $^{35}$S-labeled LS synthesized was quantitated by immunoprecipitation and gel analysis as described (24).

**$NaDodSO_4$/PAGE.** Protein extracts from whole tissue and plastids were solubilized by heating in 2% $NaDodSO_4$ containing 0.1 M 2-mercaptoethanol for 3 min at 100°C. The samples were electrophoresed in 12% polyacrylamide slab gels, using the discontinuous buffer system of Laemmli (25). Gels were stained in 50% methanol/7.5% acetic acid/0.25% Coomassie blue R-250 and destained with 20% methanol/7.5% acetic acid.

## RESULTS

**Effect of Light on the Level of Rbu$P_2$Case Activity.** The results shown in Fig. 1 confirm previous findings (6, 7) that light markedly increases the level of Rbu$P_2$Case in peas. For these experiments, plants were grown in the dark for 14 days before exposure to light. The plants grown only in the dark for 14 days had very low levels of Rbu$P_2$Case activity which remained essentially the same when the plants were kept in the dark for an additional 24 hr. On the other hand, when 14-day-dark-grown plants were illuminated, there was a dramatic increase in the rate of appearance of Rbu$P_2$Case activity over a 72-hr period. Based on a 24-hr period, the specific activity of Rbu$P_2$Case in plants kept in the dark for 14 days changed very little (<0.3 unit/day) whereas after light exposure, the specific activity increases about 60 units per day for at least 3 days after an initial lag during the first 24 hr of light (Fig. 1). To see whether the large increase in the specific activity of the enzyme was due to light activation of preexisting enzyme present in the dark-grown cells, both

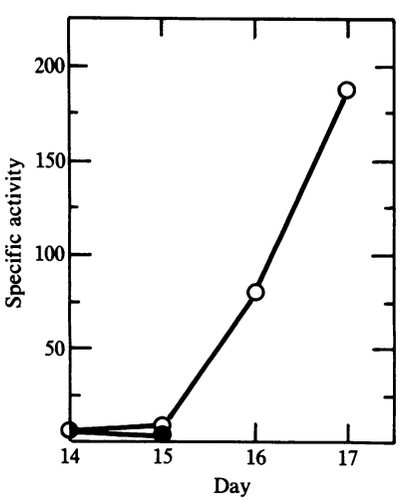


FIG. 1. The effect of light on the specific activity of Rbu$P_2$Case. Pea seedlings were grown in the dark for 14 days and then either kept in the dark (●) or exposed to light for 72 hr (○). Plants were harvested at various times and the Rbu$P_2$Case activity of cell-free extracts were determined. Specific activity is defined as nmol of $^{14}CO_2$ fixed per mg of protein per min of incubation.

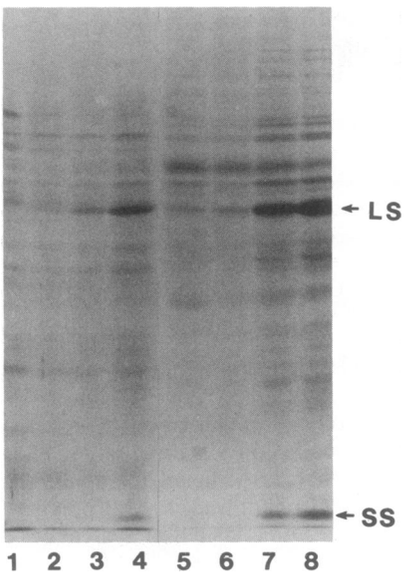


FIG. 2. NaDodSO$_4$/PAGE of proteins from dark- and light-grown pea plants. Pea seedlings were grown in the dark for 14 days and then exposed to light for various periods of time. The plant tissue was homogenized and both a cell-free extract and isolated organelles were prepared. Aliquots (50 $\mu$g of protein) were electrophoresed and the proteins were stained with Coomassie blue. Lane 1: 14 day-dark-grown total leaf extract. Lanes 2–4: leaf extract from 14-day-dark-grown plants exposed to light for 16, 32, or 48 hr, respectively. Lane 5: plastid extract from 14-day-dark-grown plants. Lanes 6–8: plastid extract from 14-day-dark-grown plants exposed to light for 16, 32, or 48 hr, respectively.

total leaf and plastid soluble proteins were extracted from dark-grown plants and dark-grown plants that had been exposed to light. The proteins were resolved by NaDodSO$_4$/PAGE and stained with Coomassie blue (Fig. 2). Two major protein bands in total leaf extracts of illuminated plants (lanes 2–4) comigrated with the LS and SS of Rbu$P_2$Case, which were virtually absent in extracts of dark-grown plants (lane 1). Since the carboxylase is located in plastids, soluble proteins from isolated organelles were examined in order to lower the background of cytoplasmic proteins and facilitate detection of low levels of LS and SS. Of four major protein bands from plastids of dark-grown plants (lane 5), one comigrated with LS. There was little or no SS detectable by staining in plastids of dark-grown plants. As with total leaf extracts, the intensity of the LS band increased dramatically (10- to 20-fold) in plastids isolated from light-grown plants and the appearance of the SS appeared to be coordinated with the onset of Rbu$P_2$Case activity (Fig. 2, lanes 6–8, and Fig. 1). The presence of significant levels of LS in etioplasts of dark-grown pea (Fig. 2, lane 5) is in general agreement with the results of Siddell and Ellis (26), who reported that LS was one of the major proteins in pea etioplasts. The results in Figs. 1 and 2 indicate that etioplasts from dark-grown plants contain detectable levels of LS but upon illumination there is a marked increase of both LS and SS. The increase in the activity of Rbu$P_2$Case after illumination is due to increased synthesis of both subunits of the enzyme.

**The Effect of Light on the Level of LS mRNA.** Previous studies indicated that exposure of pea seedlings to light resulted in an increase of LS mRNA that correlated with the chloroplast genome copy number (8, 10). We initially determined the LS mRNA levels by dot blot hybridization. In these experiments the RNA was extracted from either whole leaves or plastids isolated from dark-grown plants and dark-grown plants that had been illuminated. The RNA was fixed to nitrocellulose filters and then hybridized to $^{32}$P-labeled DNA containing LS-related sequences (see *Materials and Methods*). Table 1 shows that the amount of LS mRNA (per $\mu$g of total leaf RNA) from dark-grown plants increased only about 3-fold after the plants were shifted into the light for 72 hr. However, when the amount of LS mRNA was determined per $\mu$g of plastid RNA, little difference was observed between the dark plants and those exposed to light (Table 1).

Table 1. Exposure to light increases the amount of hybridizable LS mRNA

| | cpm/$\mu$g | | |
|---|---|---|---|
| | Hybridization to whole leaf RNA | Hybridization to plastid RNA | |
| Time in light, hr | | Exp. 1 | Exp. 2 |
| 0 (dark-grown) | 84 | 1251 | 3160 |
| 24 | 56 | 1796 | — |
| 48 | 154 | 1858 | 3160 |
| 72 | 208 | 1498 | 3280 |

RNA was extracted from plants grown in the dark and from dark-grown plants subjected to light for the times indicated. The RNA was extracted, fixed to nitrocellulose paper, and hybridized to $^{32}$P-labeled DNA containing LS sequences. Hybridizations were performed using 15 $\mu$g of whole leaf RNA and 1 $\mu$g of plastid RNA. Details are described in the text.

**Effect of Light on Plastid DNA Copy Number.** The above results could be explained if light increased the total amount of plastid RNA relative to total RNA, without any specific effect on the synthesis of the LS mRNA. In pea, it has been reported that the amount of cpDNA increases 4- to 5-fold after exposure to light (10, 27) and we have observed a similar effect. For these experiments, total pea DNA, labeled *in vivo*, was isolated from plants grown in the dark and from dark-grown plants exposed to light and then was hybridized to plasmid DNA containing cloned spinach cpDNA (see *Materials and Methods*). About 0.9% (of 54,000 cpm) and 4.8% (of 11,700 cpm) of the labeled DNA obtained from dark-grown and dark-to-light-shifted plants, respectively, hybridized to the cloned cpDNA.* Thus, in these experiments, there is about a 5-fold increase in plastid DNA sequences in plants exposed to light compared to plants grown only in the dark. This increase in genome copy number could account for the observed increase in LS mRNA after light exposure (Table 1). However, a 5-fold increase in LS mRNA appears to be insufficient to account for the very large increase in the synthesis of LS or in the specific activity of Rbu$P_2$Case observed after illumination of dark-grown plants (see Fig. 1).

***In Vitro* Translation of LS mRNA from Etioplasts and Chloroplasts.** The hybridization data (Table 1) showed that etioplasts from dark-grown plants have the same amount of hybridizable LS mRNA per $\mu$g of total plastid RNA as plastids from lighted plants. However, these experiments do not provide any information on the functional activity of the LS mRNA. To answer this question, RNA isolated from plastids from either dark-grown plants or dark-grown plants exposed to light was used as template in a highly defined *in vitro E. coli* protein synthesizing system (22, 23). The amount of LS synthesized was estimated by gel electrophoresis and quantitated by immunoprecipitation and gel analysis (24). When aliquots from the *in vitro* incubations, using plastid RNA as template, were subjected to NaDodSO$_4$/PAGE and fluorographed, each incubation yielded a radioactive band of the same mobility as LS (Fig. 3). The intensity of this band was similar whether the RNA was obtained from dark- or light-grown plants. Quantitation of the LS synthesized confirmed the results of Fig. 3. As shown in Table 2, the RNA

*These values reflect only relative amounts of DNA hybridized, since the extent of pea and spinach cpDNA cross-hybridization is not known.

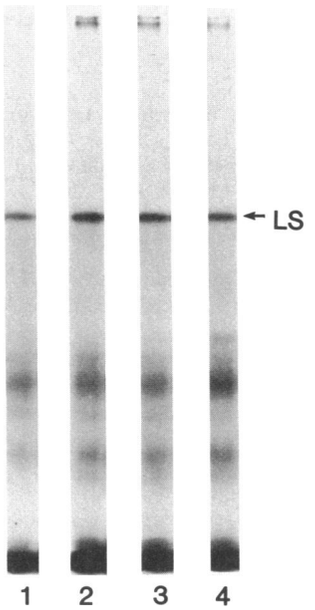


FIG. 3. *In vitro* translation products of plastid mRNA isolated from dark-grown plants and dark-grown plants exposed to light. RNA was prepared from dark-grown plants and from dark-grown plants that had been exposed to light for the indicated periods of time. The RNA (10 $\mu$g) was incubated in an *E. coli in vitro* protein synthesizing system containing [$^{35}$S]methionine (22, 23). At the end of the incubation, an aliquot was removed and subjected to NaDodSO$_4$/PAGE followed by fluorography. Lane 1: 14-day-dark-grown plants. Lanes 2–4: 14-day-dark-grown plants exposed to light for 20, 40, or 70 hr, respectively.

samples isolated from the plastids of dark- and of light-grown plants contained about the same amount of translatable LS mRNA per $\mu$g of plastid RNA.

## DISCUSSION

The mechanism by which light increases the enzymatic activity of Rbu$P_2$Case in pea involves several processes and is not fully understood. It is evident that the expression of the SS and LS genes is differentially regulated. Previous data support the view that in pea the nuclear-coded SS gene shows light regulation at the level of transcription; i.e., a specific increase in the amounts of SS mRNA (7–9). The situation with pea LS mRNA is not so clear. Shinozaki *et al.* (28) reported 20- to 30-fold increase in pea LS mRNA after illumination of dark-grown plants for 48 hr. Our results and those of Thompson *et al.* (8) show, however, that there is only about a 3-fold increase in the amount of hybridizable LS mRNA after exposure of dark-grown pea plants to light for 72 hr, which could be accounted for by a similar increase in genome copy number. These latter results are also in agreement with a recent report of Sasaki *et al.* (10) that the rate of synthesis of hybridizable LS mRNA increased 4- to 5-fold in peas after exposure to light and that this increase could, in large part, be accounted for by a similar increase in plastid genome copy number. However, a 5-fold increase in LS mRNA, although very significant, cannot explain the much larger increase in the rate of LS synthesis and Rbu$P_2$Case activity observed after plants are exposed to continuous light. These results suggest that the effect of light on LS synthesis involves an increase in gene dosage, as well as translational control. This conclusion is supported by the finding that the amount of translatable LS mRNA (per $\mu$g of plastid RNA) is essentially the same in plastids isolated from dark- or light-grown plants. Miller *et al.* (29) reported that the effect of light on *Euglena gracilis* LS synthesis may also involve translational control.

Table 2. Translatable LS mRNA in dark-grown plants and dark-grown plants exposed to light

| Time in light, hr | LS synthesized, fmol/$\mu$g of RNA |
|---|---|
| 0 (dark-grown) | 2.5 |
| 24 | 4.0 |
| 48 | 2.3 |
| 72 | 3.5 |

RNA was prepared and translated as described in the legend to Fig. 3 and *Methods and Materials*. The *in vitro* synthesized LS was quantitated by immunoprecipitation and subsequent gel electrophoresis (24). Details are described in the text and elsewhere (22–24).

At this time, we cannot explain why the LS mRNA in etioplasts from dark-grown pea is not translated efficiently *in vivo*. The LS mRNA synthesized in the dark could be part of a nontranslatable ribonucleoprotein particle, or there may be specific factors required for LS mRNA translation that are absent in dark-grown plants. Evidence for selective translation also has been come from experiments using the thylakoid and stromal fractions of chloroplasts from spinach and *Chlamydomonas reinhardtii* (30, 31). It is also possible that protein synthesis is very low in organelles from dark-grown plants, so that translation of all etioplast mRNAs, including LS mRNA, is markedly reduced. However, other studies (26, 32) have shown that in the first 48 hr of greening, there appears to be an increase of only 3-fold in the protein-synthesizing capacity of isolated plastids. A change of this magnitude may reflect the increase in total plastid RNA resulting from gene amplification and not be related to increased translational efficiency. Although translational control appears to be the most likely interpretation of the present results, another possibility is that LS synthesis occurs in dark-grown plants, but since there is little or no SS available (and thus no formation of holoenzyme), the LS is rapidly destroyed.

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