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Capacity for RNA Synthesis in 70S Ribosome-deficient Plastids of Heat-bleached Rye Leaves

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Abstract. In the leaves of rye seedlings (*Secale cereale* L.) grown at an elevated temperature of 32° C the formation of plastidic 70S ribosomes is specifically prevented. The resulting plastid ribosome-deficient leaves, which are chlorotic in light, represent a system for the identification of translation products of the 80S ribosomes among the chloroplastic proteins. Searching for the primary heat-sensitive event causing the 70S ribosome-deficiency, the thermostability of the chloroplastic capacity for RNA synthesis was investigated. The RNA polymerase activity of isolated normal chloroplasts from 22°-grown rye leaves was not inactivated in vitro at temperatures between 30° and 40° C. The ribosome-deficient plastids purified from bleached 32°-grown leaf parts contained significant RNA polymerase activity which was, however, lower than in functional chloroplasts. After application of [^{3}H]uridine to intact leaf tissues [^{3}H]uridine incorporation was found in ribosome-deficient plastids of 32° C-grown leaves. The amount of incorporation was similar to that in the control chloroplasts from 22° C-grown leaves. According to these results, it is unlikely that the non-permissive temperature (32° C) causes a general inactivation of the chloroplastic RNA synthesis in rye leaves.

Key words: Chloroplast biogenesis; RNA) – Ribosomes, plastidic – RNA (chloroplastic) – RNA polymerase – *Secale* – Temperature and RNA polymerase.

Introduction

In several higher plant species the formation of the chloroplast ribosomes exhibits a preferential high-temperature sensitivity (Feierabend and Mikus 1977). At a suitable non-permissive elevated temperature, usually between 32° C and 34° C, the accumulation of 70S ribosomes and of their rRNA in the leaves is prevented, while seedlings of such plants are still able to grow with little impairment (Feierabend and Schrader-Reichhardt 1976; Schäfers and Feierabend 1976; Feierabend 1979). However, defective plastids develop and the 70S ribosome-deficient parts of leaves grown in light are chlorotic (Schäfers and Feierabend 1976). Our investigations with rye seedlings have shown that the non-permissive high temperature appeared to interfere quite specifically with some step in the biosynthesis or assembly of the 70S ribosomes, and its detrimental effects were confined to the development of the plastids (Feierabend and Schrader-Reichhardt 1976; Schäfers and Feierabend 1976; Feierabend 1979). Proteins which have to be synthesized on 70S ribosomes were missing. However, many other chloroplast proteins which are translation products of the cytoplasmic 80S ribosomes were still synthesized and accumulated and, therefore, 70S ribosome-deficient leaves from 32° C-grown rye seedlings serve as a system for studying the role of the cytoplasm in the synthesis, transport, and assembly of chloroplast proteins (Feierabend and Schrader-Reichhardt 1976; Feierabend 1977; Feierabend and Wildner 1978; Feierabend 1979).

According to present knowledge (Bogorad 1975; Boynton et al. 1976; Harris et al. 1977; Kowallik and Herrmann 1977; Bedbrook and Kolodner 1979), the rRNA of the chloroplastic 70S ribosomes and the mRNA for some ribosomal polypeptides have to be transcribed from the chloroplast DNA. Since the chloroplastic rRNA was no longer detectable in 32° C-grown rye leaves, chloroplastic RNA synthesis appeared to be a conceivable candidate for the heat-sensitive step which might be blocked under the non-permissive temperature conditions. In addition, Brandt and Wiessner (1977) have reported that in *Euglena,* with which a heat-bleaching phenomenon

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has been observed for some time (Pringsheim and Pringsheim 1952) and which is also accompanied by a 70S ribosome-deficiency (Cohen and Schiff 1976), the chloroplastic RNA polymerase was inactivated in vitro at the bleaching temperature. Therefore, we have currently investigated whether the RNA synthesis activity of rye chloroplasts is inactivated in vitro or in vivo at the non-permissive high temperature.

Material and Methods

Plant Material and Growing Conditions. Seedlings of winter rye (*Secale cereale* L.) cv. Petkus "Kustro" were grown for 5 or 6 days, as indicated, in the light (5,000 ± 500 lx) in glass-covered plastic boxes on filter paper moistened with either distilled water or nutrient solution (only for material of Fig. 5), at either 22° or 32° C (Feierabend and Schrader-Reichhardt 1976).

Isolation of Plastids. Leaf tips were discarded and the lower parts of the leaf material (completely chlorotic tissue of 32° C-grown leaves) was surface sterilized in a 1% solution of sodium hypochlorite; then they were rinsed several times with sterilized distilled water, blotted dry, and cooled on ice. First the resulting tissue was finely minced with razor blades in 2–3 volumes of ice-cold grinding medium and then briefly and gently ground in a mortar in the presence of a small quantity of sea sand. To measure the RNA-polymerase activity, homogenization was performed in Bottomley's medium (1970), except that 0.1 M tricine-KOH buffer, pH 8.0, was used. After incorporation of [^{3}H]uridine into the intact leaves, the tissue was homogenized in the grinding medium described by Feierabend and Beevers (1972), with the following modifications: 4 mM dithioerythritol was used instead of ascorbate; ficoll was omitted; and 0.01% (w/v) bovine serum albumin was added. The homogenates were pressed through four layers of muslin and four layers of Miracloth. The sediment obtained after 20 s centrifugation at 270 *g* was discarded. The supernatant was centrifuged for 60 s (chloroplasts) or 120 s (bleached plastids) at 2,000 *g*. The resulting sediment was resuspended in grinding medium (1 ml per g initial fresh weight) and centrifuged on sucrose gradients described as gradient B by Feierabend and Schrader-Reichhardt (1976). The sucrose solutions of the gradient were contained in 50 mM tricine-KOH, pH 7.5, 1 mM EDTA and 2 mM $MgCl_2$.

Incorporation of [^{3}H]Uridine. For labeling with [^{3}H]uridine, the upper one-third of each of 60 leaves from 5-day-old seedlings was removed and the lower segments were placed with their cut bases into 0.2 ml H_2O containing $7.5 \cdot 10^5$ Bq [^{3}H]uridine. When the solution had been taken up it was replaced by sterilized H_2O. The leaves were kept for 6 h in light at the temperature where they had been grown (either 22° C or 32° C).

After termination of the labeling period the leaves were rinsed and homogenized as described above. Before the centrifugation on the sucrose gradients, the 2,000 *g* sediments were resuspended in grinding medium containing 50 µg per ml pancreas RNAase and 8 µg per ml T_1-RNAase. Then they were incubated for 20 min at 4° C in order to remove cytoplasmic RNAs contaminating the plastids at their outer surface. At the end of the ribonuclease treatment the plastids were pelleted (2 min 4,300 *g*), resuspended in fresh grinding medium (0.5 ml per g initial fresh weight), layered onto the sucrose gradients, and centrifuged, as described above.

The total content of [^{3}H] and the incorporation of [^{3}H]uridine into material precipitated by 10% trichloroacetic acid (final concentration) was determined in the various fractions obtained. Per 0.5 ml of the sucrose gradient fractions 0.2 ml of a 0.5% yeast RNA solution was added before precipitation. The precipitates were washed three times with 5% trichloroacetic acid, 80% ethanol, and finally with ether. The washed precipitates were solubilized in hyamine hydroxide or tissue solubilizer TS 1 (Koch-Light Laboratories, Ltd.) and the radioactivity was counted after the addition of Aquasol scintillation fluid. An internal standard was used for the estimation of dps.

Portions of the main plastid-containing gradient fractions were pooled, diluted with an equal volume of 50 mM Tris-HCl, pH 7.6, containing 10 mM $MgSO_4$, after which 0.5 mg ml^{-1} yeast RNA was added. After lysis by treatment with 0.2 mg ml^{-1} Proteinase K in the presence of 0.5% sodium dodecylsulfate and extraction with phenol-cresol (Parish and Kirby 1966) the nucleic acids were precipitated with two volumes of ethanol at −20° C. The dried precipitate was dissolved in 50 mM Tris-HCl, pH 7.6, containing 10 mM $MgSO_4$ and incubated with 0.1 mg ml^{-1} DNAase I (20 min 37° C). Samples of the solution were then dried on filter-paper discs (Whatman 3). The filter discs were washed with 10% trichloroacetic acid, 5% trichloroacetic acid, ethanol: ether (3:1), and ether. They were then dried and counted for radioactivity.

Analytical Methods. Enzyme activities. NADP-dependent glyceraldehydephosphate dehydrogenase (EC 1.2.1.13) and fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) were assayed as described previously (Feierabend and Schrader-Reichhardt 1976). RNA polymerase activity (EC 2.7.7.6) was assayed according to Bottomley et al. (1971) with the following procedural modifications: the assay mixture (0.25 ml) additionally contained chloramphenicol (6.25 µg per 0.25 ml) and calf thymus DNA (33 µg per assay; Bottomley et al. 1972) instead of maize DNA; and the reaction was started by adding 185 KBq 5,6-[^{3}H]UTP (for Figs. 1 and 2: 74 KBq per assay). If not otherwise indicated, the incubation time was 30 min at 37° C. After termination of the reaction 1 ml of a 0.2% yeast RNA solution was added per assay. The acid insoluble precipitates were centrifuged, washed (2·5% trichloroacetic acid + 0.005% UTP, 3·5% trichloroacetic acid in 50 mM sodium pyrophosphate, 1·80% ethanol, 1·ether), and solubilized in hyamine hydroxide or tissue solubilizer TS-1. The samples were added to an Aquasol scintillation fluid and the radioactivity was counted.

Chlorophyll was estimated according to Whatley and Arnon (1963). Sucrose concentrations were determined with a Zeiss Abbé-refractometer, Model A.

Results

RNA Polymerase Activity of Isolated Chloroplasts. Gradient-purified intact chloroplasts obtained from 22° C-grown green rye leaves which were broken by osmotic shock in the assay medium possessed RNA polymerase activity. The in vitro incorporation of [^{3}H]UTP into trichloroacetic acid precipitable material required the presence of the three other nucleotides, ATP, CTP, and GTP, was sensitive to ribonuclease treatment, and, over a wide range, was proportionate to the amount of chloroplast material added to the assay (Fig. 1). The incorporation of [^{3}H]UTP was most rapid during the first 20 min and ceased after about 45 min of incubation (Fig. 2). Between 25° C and 42° C the rate of [^{3}H]UTP incorporation increased but had not yet reached its optimum so

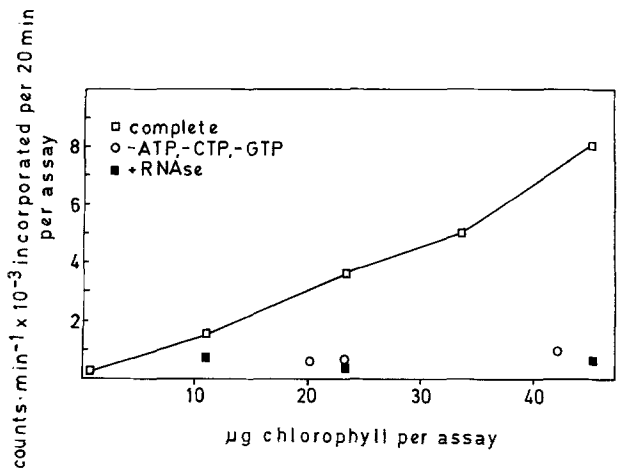

Fig. 1. Dependence of the RNA polymerase activity of a preparation of isolated intact chloroplasts from 22° C-grown rye leaves on the presence of nucleotides and on the amount of chloroplast material added to the assay. Incubation time was 20 min at 25° C. ○ ATP, CTP and GTP were omitted from the assay; ■ the assays additionally contained 12.5 µg pancreas ribonuclease

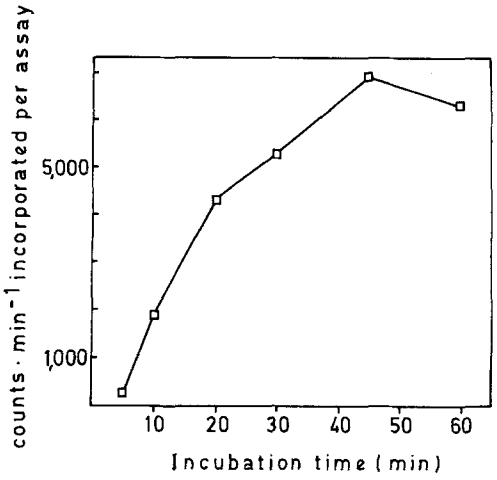

Fig. 2. Time course of UMP incorporation at 25° C by isolated chloroplasts from 22° C-grown rye leaves in an assay for RNA polymerase activity

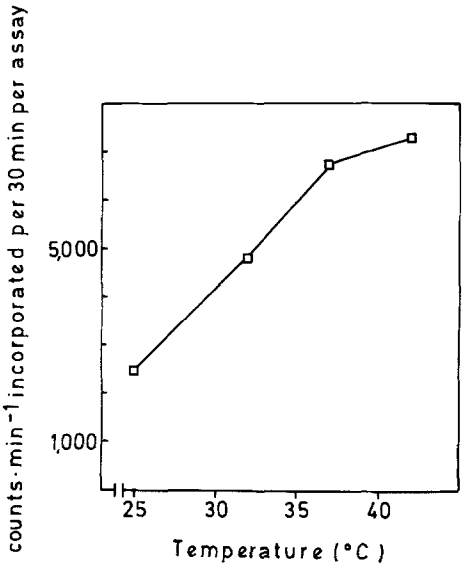

Fig. 3. Temperature dependence of the RNA polymerase activity of isolated rye chloroplasts

that there was clearly no in vitro inactivation of the chloroplast RNA polymerase activity at temperatures around 32° C (Fig. 3). Therefore the RNA polymerase assays in the following were performed at 37° C.

Comparison of Chloroplasts and Bleached Ribosome-deficient Plastids. Chloroplasts and the ribosome-deficient plastids of completely bleached 32° C-grown rye leaves were isolated by short centrifugations on discontinuous sucrose gradients (Fig. 4a and b), where the intact plastids were separated from broken plastids because of their higher density, and from contaminating mitochondria or peroxisomes because of their higher sedimentation rate. The main peak of the soluble marker enzyme fructosebisphosphate aldolase which was used to identify the location of intact plastids was, both for the chloroplasts from 22° C-grown leaves and for the ribosome-deficient plastids from 32° C-grown leaves, closely accompanied by a peak of RNA polymerase activity, indicating the presence of the latter in the bleached plastids as well (Fig. 4b). However, relative to equal amounts of tissue fresh weight, less RNA polymerase activity was present in the bleached plastids than in the chloroplasts. In order to avoid differences resulting from unequal organelle yields obtained during homogenization and isolation from the different types of leaf tissue, preparations with almost equal yields of the marker enzyme fructosebisphosphate aldolase are compared in Fig. 4. Relative to the aldolase activities present in the intact plastid fractions, the ribosome-deficient plastids exhibited only about 40% of the RNA polymerase activity found in the chloroplasts of 22° C-grown leaves.

[^{3}H]Uridine Incorporation into Chloroplasts and Heat-Bleached Plastids. In order to obtain some, though still rough, information on whether the ribosome-deficient plastids were also capable of RNA synthesis in situ at 32° C, leaves grown at either 22° C or 32° C were excised and allowed to take up and incorporate [^{3}H]uridine. Subsequently the plastids were isolated and treated with ribonuclease to remove contaminating cytoplasmic RNAs. Incorporation into

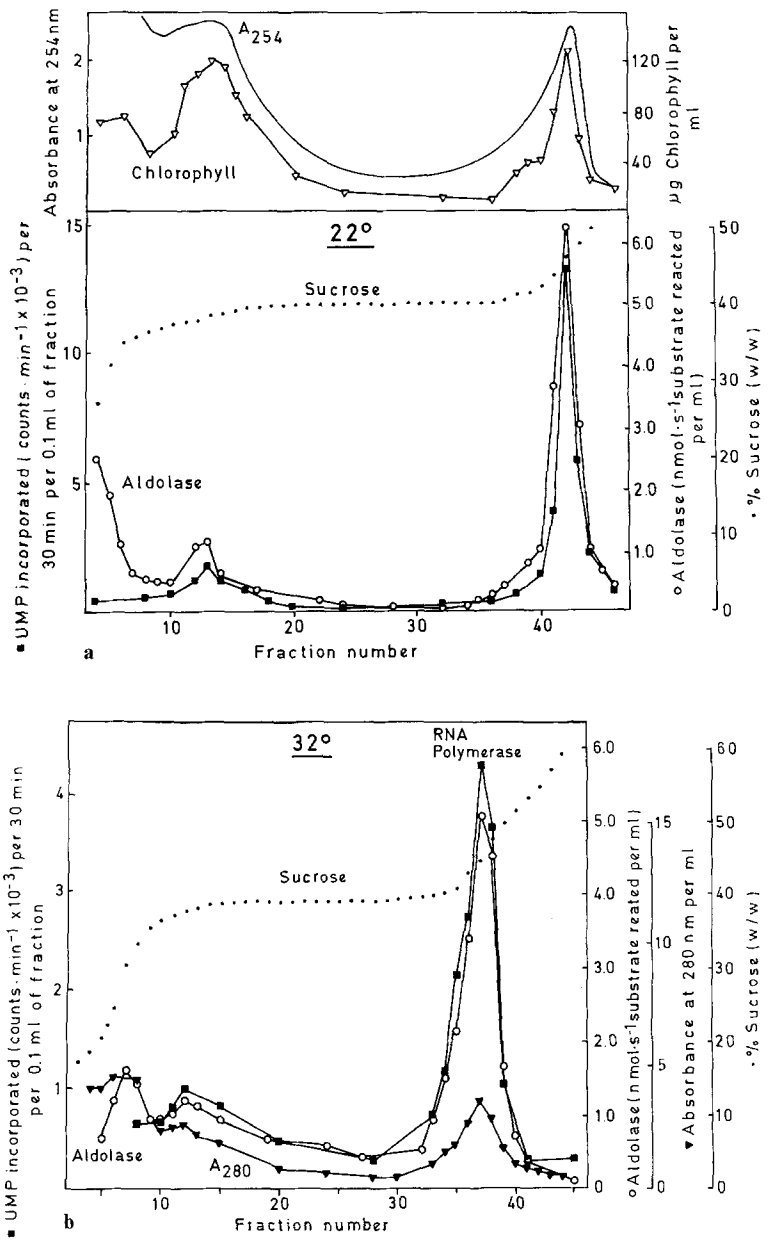

Fig. 4a and b. Sucrose gradient isolation of unbroken chloroplasts from 22° C-grown rye leaves (**a**) and of unbroken plastids from chlorotic parts of 32° C-grown rye leaves (**b**). — absorbance at 254 nm, ▼ absorbance at 280 nm; ○ fructose-1,6-bisphosphate aldolase activity; ■ RNA polymerase activity; ▽ chlorophyll content; · sucrose concentration

plastids was further analyzed after sucrose gradient centrifugation. With regard to soluble marker enzymes such as NADP-glyceraldehyde-phosphate dehydrogenase or fructose-1,6-bisphosphate aldolase, the ribonuclease treatment did not significantly influence the integrity of the isolated unbroken plastids since the contents of the marker enzyme activities remained unchanged. Under the experimental conditions used, about 3% (32° C-grown leaves) or 5% (22° C-grown leaves) of the [^{3}H]uridine taken up by the leaves were incorporated into trichloroacetic acid precipitable material, and between 4% (32° C-grown leaves) and 7% (22° C-grown leaves) of the incorporated radioactivity were located in the crude plastid fractions after the treatment with ribonuclease. Further analysis of the ribonuclease-treated crude plastid fractions by sucrose gradient centrifugation (Fig. 5) showed that, both for the preparations from green 22° C-grown and for those from chlorotic 32° C-grown leaves, the only major peak of precipitable radioactivity on the gradients was located in the position of intact chloroplasts or bleached plastids, respectively, which were indicated by the soluble marker enzymes NADP-glyceraldehydephosphate dehydrog-

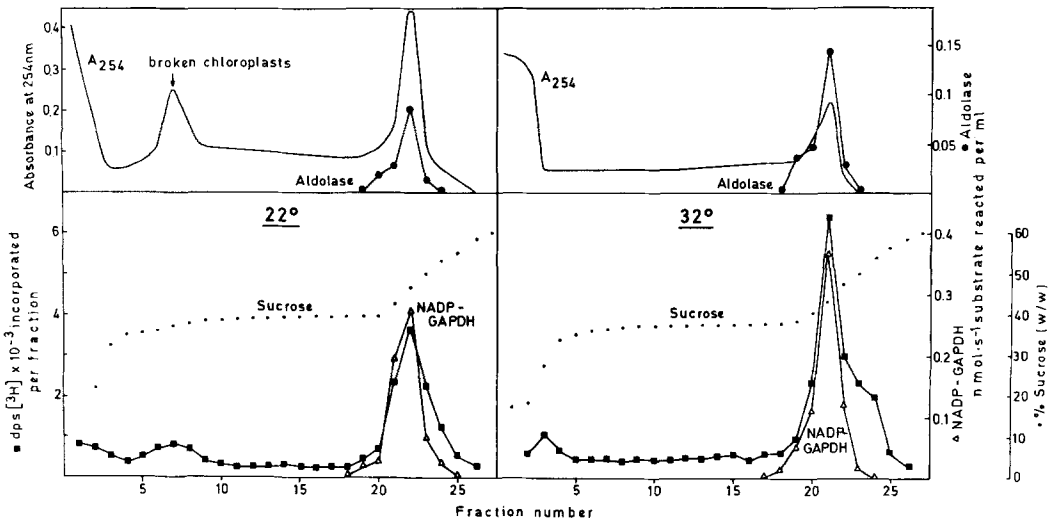

Fig. 5. In vivo incorporation of [^{3}H]uridine into chloroplasts from 22° C-grown leaves and bleached plastids from chlorotic parts of 32° C-grown rye leaves. Sucrose gradient isolation of intact plastids. ■ trichloroacetic acid precipitable radioactivity; ● fructose-1,6-bisphosphate aldolase; ▲ NADP-dependent glyceraldehydephosphate dehydrogenase; —— absorbance at 254 nm; · sucrose concentration

enase and fructosebisphosphate aldolase. Only little radioactivity was, for instance, associated with broken chloroplasts. Thus, the results indicate the presence of [^{3}H]uridine incorporation in the ribosome-deficient plastids of 32° C-grown chlorotic leaf tissue. The amount of incorporation into these plastids was, although it varied to some extent among different preparations, of quite a similar order of magnitude relative to equal leaf material used for the preparations or per yield of plastidic marker enzymes, as for the functional chloroplasts. In addition, when samples of the gradient fractions containing either intact chloroplasts or bleached plastids were withdrawn and the RNA was extracted and separated from the protein and DNA, similar amounts of radioactivity were retained in the RNA for both growth conditions.

Discussion

It is now established that chloroplasts contain DNA which contains, for instance, also the information for the chloroplastic rRNA, and that they are capable of transcription (Ellis 1976; Kowallik and Herrmann 1977; Bedbrook and Kolodner 1979). An RNA polymerase activity has been found in, or purified from, isolated chloroplasts, particularly from spinach, maize, peas, and *Euglena* (Bottomley et al. 1971, 1972; Hartley and Ellis 1973; Smith and Bogorad 1974; Bohnert et al. 1976; Schiemann et al. 1978). Brandt and Wiessner (1977) have reported that the chloroplastic RNA polymerase of an heat-sensitive strain of *Euglena* was inactivated in vitro at temperatures above 30° C. Such a general inactivation of the chloroplastic RNA polymerase activity in the non-permissive temperature range would stop transcription of the plastidic rRNA as well as that of some genes for ribosomal polypeptides and, thus, represents a conceivable reason for the heat-induced chloroplast ribosome-deficiency and bleaching observed in *Euglena* and in several higher plants. We have therefore investigated the thermostability of the plastidic RNA synthesis in rye leaves.

RNA polymerase activity was present in gradient-purified chloroplasts from 22° C-grown rye leaves. Though the enzyme was not further purified and its molecular identity has not been documented and distinguished from the nuclear RNA polymerases, it is strongly anticipated, from the isolation procedure for the intact chloroplasts, that non-chloroplastic contaminations are rather low, if present at all. During the gradient centrifugation employed, bacteria, nuclei, or larger nuclear fragments would have sedimented through the intact chloroplast layer because of their high densities, while broken chloroplasts, mitochondria, and peroxisomes would remain in the upper part of the gradient because of their lower density or their lower sedimentation rates, respectively. Thus, trapping of non-chloroplastic enzyme activities in the unbroken chloroplast fraction was found to be negligable, particularly when Ficoll was not present in the homogenization medium (Feierabend and Schrader-Reichhardt 1976; Feierabend 1977).

The present results clearly indicated that, for rye chloroplasts, the non-permissive temperature neither inactivated the activity of chloroplastic RNA poly-

merase in vitro nor prevented its appearance in the bleached plastids during growth at 32° C. In accordance with corresponding results from inhibitor experiments (Surzucki et al. 1970; Ellis and Hartley 1971), the presence of RNA polymerase activity in the ribosome-deficient plastids also indicated that the enzyme is presumably synthesized on cytoplasmic ribosomes. However, since the RNA polymerase activity of ribosome-deficient plastids from 32° C-grown leaves was lower than that of functional chloroplasts, the possibility exists that some specific RNA, e.g., the rRNA, failed to be synthesized at the non-permissive temperature.

The incorporation of labeled uridine into the defective plastids of intact bleached leaf tissues at 32° C shows that some plastidic RNA synthesis also seem to be active in vivo at 32° C. This finding is, of course, preliminary in so far as the nature of the products synthesized in the plastids at 32° C in vivo is unknown and the occurrence of contaminations with RNA from the rest of the cell, though presumably low because of the strong ribonuclease treatment of the isolated plastids, cannot be completely excluded. It can also not be excluded that, in vivo, newly synthesized RNA was imported into the defective plastids, whereas such an RNA transport is so far unknown. Although the similarities in the amounts of uridine incorporation into the ribosome-deficient plastids at 32° C and into the chloroplasts at 22° C are a strong indication that the labeling in the defective plastids cannot only be due to some negligible contamination, both conditions cannot be adequately compared in a quantitative way. It has not yet been precisely assessed how the temperature difference of 10° C influenced the rates of uptake and incorporation of uridine, and the sizes of the internal uridine pools in the chlorotic 32° C-grown leaf tissue may well be quite different from those in the green control leaves.

Although several unsolved questions remain, our results do not support the possibility of a general inactivation of the chloroplastic RNA synthesis under the non-permissive temperature conditions as a conceivable cause for the 70S ribosome-deficiency of rye leaves. Also, a major interference with, or complete elimination of, the chloroplastic DNA which has been described for heat-bleached *Euglena* (Edelman et al. 1965; Ray and Hanawalt 1965) was not observed in the 32° C-grown rye leaves (Herrmann and Feierabend 1980) and must, therefore, be excluded as a possible reason for the inability of the rye plastids to form ribosomes at 32° C. It remains to be investigated whether the non-permissive temperature blocks a more specific biosynthetic step involved in the ribosome formation, such as the synthesis or processing of the rRNAs, or the synthesis or transport of some ribosomal polypeptide, or whether it prevents the assembly of the different building blocks in the ribosomal subunits. An interference of temperature conditions with the assembly of ribosomal structures is, for instance, well known for several cold-sensitive ribosomal mutations of bacteria (Geyl et al. 1977; Isono et al. 1977).

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