Phytochemistry, 1975, Vol. 14, pp. 609–612. Pergamon Press. Printed in England.

EFFECTS OF INHIBITORS ON GROWTH AND RIBOSOMAL-RNA SYNTHESIS IN CULTURED SPINACH LEAF DISCS

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(*Revised Received* 10 *September* 1974)

Key Word Index—*Spinacea oleracea*; Chenopodiaceae; ribosomal-RNA; chloroplasts; rifamycin SV; rifampicin; streptolydigin; chloramphenicol; lincomycin, inhibition.

Abstract—In single isotope labelling experiments it was found that rifamycin SV (100 μg/ml) but not rifampicin (100 μg/ml) inhibited cytoplasmic ribosomal-RNA synthesis. Dual-isotope labelling experiments established that rifamycin SV inhibited light-stimulated chloroplast ribosomal-RNA synthesis to the same extent. Light-stimulated chloroplast ribosomal-RNA synthesis was specifically inhibited by streptolydigin (50 μg/ml), lincomycin (100 μg/ml) and chloramphenicol (10 μg/ml).

INTRODUCTION

Using a double-isotope-labelling technique, we have recently obtained results which indicate that the mechanism of light-stimulated chloroplast ribosomal-RNA synthesis in cultured spinach leaf discs differs from that of cytoplasmic ribosomal-RNA synthesis and in some respects resembles that which occurs in prokaryotes [1]. Other workers using different methods have arrived at essentially the same conclusion with respect to chloroplast ribosomal-RNA synthesis in both *Nicotiana rustica* [2] and spinach [3].

We have now examined the effects of a range of prokaryote-specific inhibitors of ribosomal-RNA synthesis on light-stimulated chloroplast ribosomal-RNA synthesis in cultured spinach leaf discs. As the inhibition of chloroplast ribosome synthesis must ultimately affect chloroplast development and replication, the effect of these inhibitors on the growth of leaf discs was also measured. The results presented here indicate that the inhibitory effects of some of these antibiotics in leaf cells are different from those reported for bacterial cells.

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RESULTS

Rifamycin SV and rifampicin

Rifamycin SV and rifampicin [3-(4-methylpiperazinyliminomethyl)rifamycin SV] are antibiotics which inhibit initiation of RNA transcription in bacterial systems by combining with RNA polymerase. Since they are light-sensitive antibiotics, experiments to study their effect on the light-dependent process of chloroplast ribosomal-RNA synthesis are a compromise between complete light inactivation of the inhibitors and failure to achieve the light-dependent synthesis of chloroplast ribosomal-RNA.

Effects on disc growth. The approach used in these experiments was that used previously in which the discs were pre-grown in the dark before transferring them to the light [4]. Figure 1 shows the effects of rifamycin SV and rifampicin on the growth of leaf discs when cultured in darkness for 3 days and then transferred to alternating day/night (14/10 hr) illumination for a further 4 days. At a concentration of 100 μg/ml rifamycin SV reduced disc fresh weight, cell size, chloroplast number and chloroplast size. In contrast, at the same concentration rifampicin had little effect on disc growth.

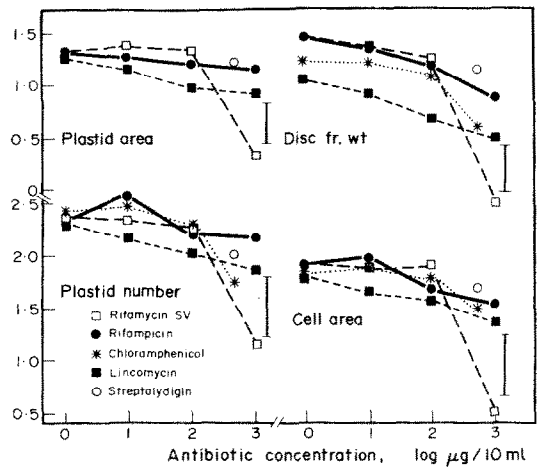

Fig. 1. Effects of antibiotics on growth of spinach leaf discs cultured in nutrient-agar. □, Rifamycin SV; ●, rifampicin (3 days dark, 4 days light); ✳, chloramphenicol; ■, lincomycin; ○, streptolydigin (7 days light). Daylength 14 hr at 21 500 lx, temp 26°. Data for plastid number, plastid area, disc fr wt and cell area are $\log_{10}$. L.S.D's are at 5% levels.

Effects on ribosomal-RNA synthesis. In single isotope incorporation studies, cytoplasmic ribosomal-RNA synthesis in leaf discs cultured in the presence of 100 μg/ml rifamycin SV was strongly inhibited (39% of control), but was unaffected by 100 μg/ml rifampicin (101% of control). The effect of rifamycin SV (100 μg/ml) on light-stimulated chloroplast ribosomal-RNA synthesis is shown in Fig. 2. Although the total incorporation into ribosomal-RNAs in this dual labelling experiment was reduced to *ca* 15% of that achieved in the absence of rifamycin SV, the ratio scans showed that light-stimulated chloroplast ribosomal-RNA synthesis did occur in the presence of rifamycin SV, since two peaks of MWs 1·04 and 0·56 × 10^6 daltons were present in the ratio light/dark scan (Fig. 2b) that were absent in the ratio dark/dark scan (Fig. 2a).

Streptolydigin

Streptolydigin has been reported to be a specific inhibitor of RNA polymerase in bacteria, and is effective by blocking phosphodiester bond formation (chain elongation) during transcription [5]. The effect on light-stimulated chloroplast ribosomal-RNA synthesis measured by dual labelling is shown in Fig. 3. The light-stimulated synthesis of 1·04 and 0·56 × 10^6 daltons chloroplast ribosomal-RNAs which occurred in the absence of streptolydigin (compare Fig. 3b with Fig. 3a), was inhibited by the presence of 50 μg/ml of the antibiotic (Fig. 3c), although it did not markedly reduce disc growth (Fig. 1).

Lincomycin and chloramphenicol

The inhibitory action of these antibiotics on protein synthesis on 70s ribosomes in both prokaryotes and chloroplasts is well known. They also promote secondary inhibition of ribosomal-RNA synthesis [6,7]. In dual-labelling experiments, lincomycin (100 μg/ml) abolished completely the light-stimulated synthesis of chloroplast ribosomal-RNAs (Fig. 3d). This inhibitor also reduced

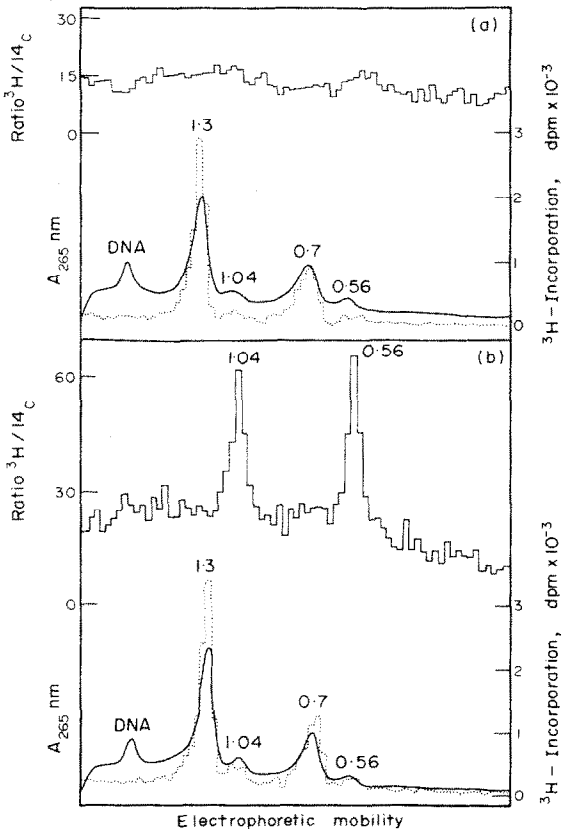

Fig. 2. Effect of rifamycin SV on light-stimulated chloroplast ribosomal-RNA synthesis. The culture conditions and dual-labelling technique are described in the text. Rifamycin SV was added to all four sets of leaf discs. The nucleic acids were extracted, fractionated by electrophoresis on polyacrylamide gels, and the ^{14}C and ^3H levels in 1 mm slices of the gel determined. For ease of comparison the histogram showing [^3H]-uridine/[^{14}C]-uridine incorporation ratio in the gel slices for each treatment has been plotted with the [^3H]-uridine (– – –) and UV-absorption profile (——) for that treatment, whilst the [^{14}C]-uridine incorporation profiles have been omitted. Ribosomal-RNA peaks are identified according to their MWs (× 10^6 daltons).

disc growth, the effect increasing with concentration (Fig. 1). Chloramphenicol (10 μg/ml) inhibited light-stimulated synthesis of 1·04 and 0·56 × 10^6 daltons RNAs in dual-labelling experiments, causing depressions to appear in the ratio profile at these points and also at the top and towards the bottom of the gel (Fig. 3e). At this concentration, chloramphenicol also markedly depressed disc growth, reducing both cell size and chloroplast numbers per cell (Fig. 1).

DISCUSSION

Rifampicin and rifamycin SV are potent and highly specific inhibitors of RNA polymerase in prokaryote systems. However, their reported effects on chloroplast ribosomal-RNA synthesis in algae and higher plants differ quite markedly. Thus rifampicin has been reported to specifically inhibit *in vivo* chloroplast ribosomal-RNA synthesis in *Chlamydomonas reinhardi* [8,9], *Chlorella* [10] and *Euglena gracilis* [11]. Bogorad and Woodcock [12] have reported that rifamycin SV but not rifampicin interferes with chlorophyll biosynthesis in regreening corn plants and that both antibiotics inhibit light-stimulated RNA synthesis. However, Bottomley *et al.* [13] using corn, radish and spinach found no specific effect of rifampicin or rifamycin SV on chloroplast ribosomal-RNA synthesis even at concentrations as high as 400 μg/ml. Our results with these inhibitors in spinach indicate that they have different effects. At a concentration of 100 μg/ml rifampicin does not have a marked effect on the growth of leaf discs (Fig. 1) and does not affect cytoplasmic ribosomal-RNA synthesis. Rifamycin SV at the same concentration is inhibitory to both overall growth (Fig. 1) and ribosomal-RNA synthesis. The possibility that rifampicin is excluded by the cell membrane and is unable to gain access to the intracellular matrix was not eliminated in our experiments, nor was the possibility that it may exert more marked effects at higher concentrations.

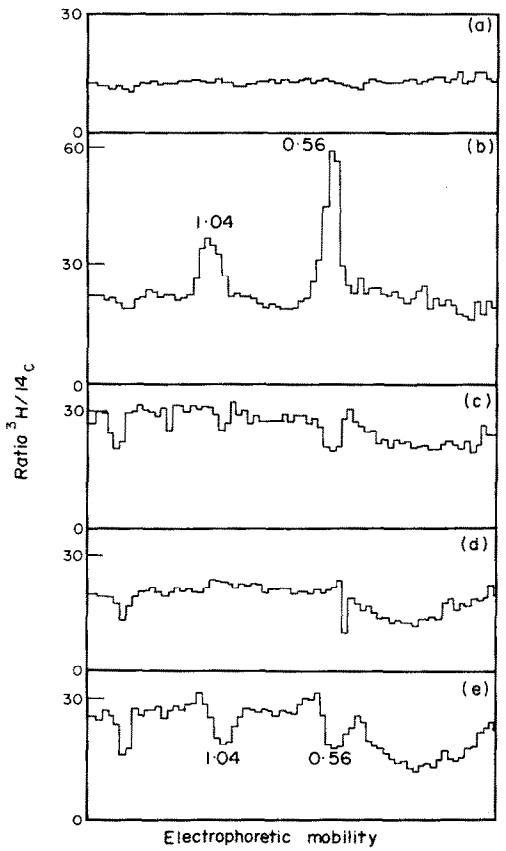

Fig. 3. Effect of antibiotics on light-stimulated chloroplast ribosomal-RNA synthesis in dual-labelled spinach leaf discs. For ease of comparison only the [^{3}H]-uridine/[^{14}C]-uridine incorporation profiles are shown. (a) Dark control, DD extract, no antibiotic treatment; (b) light control, LD extract, no antibiotic treatment; (c) LD + streptolydigin treatment 50 μg/ml; (d) LD + lincomycin treatment 100 μg/ml; (e) LD + chloramphenicol treatment 10 μg/ml. Chloroplast ribosomal-RNA peaks are labelled according to their MWs 1 × 10^6 daltons).

The inhibitory action of rifamycin SV is interesting in that it inhibited the synthesis of both cytoplasmic and chloroplast ribosomal-RNAs to the same extent. In the dual-labelling experiment (Fig. 2) total isotope incorporation into ribosomal-RNAs (mainly cytoplasmic) was only 15% of that which occurred in the absence of rifamycin SV. Because of the low levels of incorporation, no accurate assessment of the effect of rifamycin SV on light-stimulated chloroplast ribosomal-RNA synthesis can be made from single isotope incorporation data (i.e. ^{3}H-incorporation, Fig. 2). However, the ratio of ^{3}H to ^{14}C-incorporation (Fig. 2) shows both that light-stimulated chloroplast ribosomal-RNA synthesis occurred and that its amount relative to that of cytoplasmic ribosomal-RNA synthesis was similar to that which occurred in the absence of rifamycin SV (Fig. 3b).

We cannot explain the lack of specificity in the strongly inhibitory action of rifamycin SV on ribosomal-RNA synthesis in these leaf discs. However, it should be noted that a number of inhibitors of

RNA synthesis when tested on *in vivo* and *in vitro* systems isolated from higher plants have failed to reproduce the specificities that they previously exhibited in other eukaryotic and prokaryotic systems [13].

One such inhibitor is the antibiotic streptolydigin. Bottomley *et al.* [13] found that streptolydigin was equally inhibitory to RNA synthesis by both isolated chloroplasts and nuclei of spinach and pea. Figure 3 indicates that this was not the case *in vivo* for spinach leaf discs. In our experiments, streptolydigin inhibited the light-stimulated synthesis of chloroplast ribosomal-RNA but was without effect on cytoplasmic ribosomal-RNA synthesis.

The inhibitory actions of both lincomycin and chloramphenicol on chloroplast RNA synthesis have been reported previously [6,14,15]. The results described here are in complete accord with previous findings. Furthermore we suggest that the depressions in the ratio profile of the chloramphenicol-treated discs indicate that not only was light-stimulated ribosomal-RNA synthesis inhibited, but also some or possibly all, of the low-level synthesis of chloroplast ribosomal-RNA which continues in darkness, was also inhibited.

EXPERIMENTAL

Plant material. American round-seeded spinach (*Spinacea oleracea*) plants were cultivated, and selected leaf tissue was harvested, sterilized and cultured on nutrient agar as described previously [1].

Isotopes and antibiotics. Aq. solns of uridine-H3(G), uridine-C14(U) (Radiochemical Centre, Amersham, U.K.) rifampicin, rifamycin SV (Calbiochem), streptolydigin, lincomycin (Upjohn) and chloramphenicol (Sigma) were filter sterilized before addition to the agar cultures.

Chloroplast counts and cell and chloroplast area measurements were performed as previously described [16].

Dual-labelling of leaf discs. The technique as described by Detchon and Possingham [1] was used. For experiments with antibiotics, the solns were either added to all 4 plates or alternatively only to that plate which subsequently received [^{3}H]-uridine in the light. In the latter case, the DD treatments were omitted altogether. All antibiotic treatments were given in darkness 1 hr prior to the addition of radioisotopes.

Extraction and electrophoresis of nucleic acids and determination of radioactivity in dual-labelled gels were also as described previously [1].

Acknowledgements – The authors wish to acknowledge the considerable help of Mr. C. J. Brien with developing the computer program necessary for analyzing and plotting the results of the dual-labelling experiments. The competent technical assistance of Mrs. C. P. N. Hohnen, Miss J. W. Smith and Mrs. R. Luters is also acknowledged.

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