Effects of Cycloheximide and Chloramphenicol on the Multiplication of Tobacco Necrosis Virus

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SUMMARY
Infectivity studies have shown that the multiplication of tobacco necrosis virus can be inhibited by cycloheximide but not by chloramphenicol, and that it is likely that cycloheximide inhibits the synthesis of at least two proteins necessary for multiplication.

INTRODUCTION
In eukaryotic plant cells the ribosomes which occur in the cytoplasm differ in many respects from those which occur in the plastids. For example, protein synthesis on the former can be inhibited in vitro and in vivo by cycloheximide but not by chloramphenicol and vice versa (Lamb, Clark-Walker & Linnane, 1968; Hoober, Siekevitz & Palade, 1969; Hoober & Blobel, 1969; Smillie & Scott, 1969; Ellis, 1969; Graham et al. 1970). Therefore, a previous report that the multiplication of tobacco mosaic virus (TMV) can be inhibited by cycloheximide, but not by chloramphenicol suggests that protein synthesis on cytoplasmic but not plastid ribosomes is essential for the replication of TMV (Zaitlin, Spencer & Whitfield, 1968). We have found that the multiplication of an isometric plant virus, tobacco necrosis virus (TNV), in dark grown Mung bean hypocotyls can be inhibited also by cycloheximide but not by chloramphenicol. Also, that cycloheximide has a differential effect on the accumulation of virus nucleoprotein and virus RNA at various times after inoculation. During the progress of this work similar studies on tobacco rattle virus were reported (Harrison & Crockatt, 1970).

METHODS

Virus. An inoculum of TNV, strain D (Babos & Kassanis, 1963) was propagated in the leaves of French beans var. Prince for 2 days at 23 °C under conditions of high humidity. Infective RNA was prepared from leaves by homogenization with 2 vol. of phenol, saturated with water, and 1 vol. of 0.06 M-phosphate buffer, pH 7.0, at 2 °C. The emulsion was separated by centrifuging, the aqueous layer decanted and phenol removed from it by partition against diethyl ether. The preparation was divided into small portions and stored at −45 °C.

Mung beans. Seeds of Phaseolus aureus Roxb. were germinated in the dark at 28 °C to 30 °C on a pad of blotting paper and non-absorbent cotton wool wetted with distilled water. When the beans had grown 2.5 to 3.5 cm in length, approximately 44 to 48 h from the start of imbibition, the hypocotyls were inoculated manually with infective RNA and Celite. Between 45 and 60 min after inoculation the uppermost 1.0 to 1.5 cm of the hypocotyl, excluding the hook region, was excised and the segments rinsed with distilled water.
Batches of from 10 to 20 segments were transferred to 50 mm diameter plastic Petri dishes containing 2 ml 0.01 M-phosphate buffer, pH 6.5, with or without inhibitors. A disc of filter paper was placed above and below the segments to increase the area of contact between the liquid and the hypocotyls. From the beginning of imbibition, Mung beans were maintained in the dark except during inoculation and excision of the hypocotyls (15 to 45 min). Hypocotyl segments harvested during the course of an experiment to be used subsequently for determinations of infectivity were rinsed with distilled water and frozen at −35 °C or in liquid nitrogen. Extracts were prepared later from all samples at one time.

**Infectivity assay.** The infectivity of virus in sap was extracted by homogenizing 1 g fresh weight of hypocotyl segments with 1 ml of 0.06 M-phosphate buffer pH 7.0 at room temperature. Extracts were clarified by centrifuging at 10000 g for 10 min and dialysed against 0.06 M-phosphate buffer pH 7.0. Infective RNA was extracted by homogenizing 1 g fresh weight with phenol and phosphate buffer as described before. The relative infectivity of the preparations was determined by inoculating the primary leaves of French beans var. Prince, kept dark for the previous 36 h, in an incomplete block randomization so that each preparation was inoculated to a minimum of eight half-leaves. Celite was present in all inocula.

**Assay of chloramphenicol.** Hypocotyls (2.5 g fresh weight) were homogenized with 1 ml of 0.1 M-phosphate buffer pH 7.0, the extracts clarified by straining through muslin and applied to 6 mm diameter discs of filter paper (Whatman No. 1). Excess liquid was allowed to drain from the discs which were laid on nutrient agar seeded with Bacillus subtilis strain T3. The diameter of the zones of inhibition of bacterial growth were compared with those produced by samples of known concentrations of chloramphenicol in the same buffer (Harrigan & McCance, 1966).

**Assay of chlorophylls.** Crude mixtures of chlorophylls were extracted from hypocotyls by treatment with hot methanol (58 to 60 °C) and relative concentrations estimated by extinction at 654 nm.

**Incubation with [3H]-leucine.** Hypocotyl segments (0.5 g) were incubated with 2 ml 0.01 M-phosphate buffer pH 6.5 containing [3H]-L-leucine (5 μCi/ml), with or without inhibitor for 30 min. The medium was removed, the segments rinsed with distilled water, blotted dry and homogenized with 2 ml 0.01 M-phosphate buffer, pH 7.0, at 2 °C. The extract was clarified by centrifuging at 10000 g for 10 min and 0.1 ml portions of the supernatant fluid added to 0.4 ml of ice-cold 10 % (w/v) trichloroacetic acid. The precipitate was collected on a glass fibre disc, washed three times with 5% (w/v) trichloroacetic acid containing 1% (w/v) DL-leucine, rinsed with ethanol and dried. Discs were placed in vials containing 10 ml of a scintillant comprising 5 g PPO and 0.5 g dimethyl POPOP per l of toluene and the radioactivity counted in a Packard Tricarb Liquid Scintillation spectrometer (Model 3320).

**Storage in liquid nitrogen.** When preparations of infective RNA, or infective sap made from hypocotyls immediately after harvest were compared with similar preparations made from samples of the same tissue frozen in liquid nitrogen for periods from a few minutes to several weeks, no differences beyond the usual experimental variability were found.

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**RESULTS**

**Virus multiplication**

The rate of multiplication of TNV in Mung bean hypocotyls varied with temperature (Fig. 1). A temperature of 23 °C was chosen for incubation in subsequent experiments because it allowed an earlier and higher maximum infectivity than at 17 °C or 29 °C. Excision
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Fig. 1. Infectivity of virus and RNA extracted from Mung bean hypocotyls which had been inoculated with TNV-RNA and maintained at 29 °C (■ ■), 23 °C (▲ ▲ and △ △) or 17 °C (○ ○); --- virus, ---- RNA.

Fig. 2. Infectivity of virus extracted from Mung bean hypocotyls 24 h after inoculation with TNV-RNA and incubation with cycloheximide from 2 h (□ □), 12 h (■ ■) or chloramphenicol from 2 h (○ ○), 12 h (● ●).
Effect of chloramphenicol and cycloheximide on virus multiplication

Exposure of infected hypocotyls to cycloheximide (1 to 100 μg/ml) from 1/4 to 24 h after inoculation inhibited the accumulation of virus nucleoprotein, whereas exposure to chloramphenicol (10 to 1000 μg/ml) did not (Fig. 2). It is unlikely that chloramphenicol failed to penetrate the tissue because if Mung beans or excised hypocotyls were incubated with it at concentrations of 1 to 1000 μg/ml, in the light, decreases occurred in the amount of chlorophyll synthesized, the percentage of the plants in which the plumular hook uncurled, the extent of hypocotyl elongation and the fresh and dry weights, relative to controls maintained in buffer. Further, the concentration of chloramphenicol in hypocotyls incubated in solutions (from 50 to 1000 μg/ml) for 3 h was identical with that in the surrounding medium if assayed by the inhibition of bacterial growth. When cycloheximide (1 to 100 μg/ml) was included in inocula the apparent infectivity of sap or RNA was decreased. The effect was not correlated with the duration of exposure and full infectivity was restored after removal of the inhibitor by dialysis (Fig. 3).

Irrespective of the time after inoculation at which infected hypocotyls were exposed to cycloheximide (100 μg/ml) the accumulation of virus nucleoprotein was quickly inhibited...
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Fig. 4. Infectivity of virus extracted from excised Mung bean hypocotyls incubated with cycloheximide (100 µg/ml) from 1 h (a), 12 h (b), 18 h (c) and 24 h (d) or with buffer (e) or from the hypocotyl region of intact Mung beans (f), after inoculation with TNV-RNA. Bars indicate duration of exposure to cycloheximide.

Fig. 5. Infectivity of virus, ---, or RNA, ----, from excised hypocotyls incubated in buffer ○---○ and ●---●, or cycloheximide (100 µg/ml) □---□ and ■---■, from 9 h after inoculation with TNV-RNA.
Table 1. The effect of exposure to cycloheximide (50 μg/ml) from 12 h after inoculation on the amount of [3H]-leucine incorporated into an acid insoluble form by infected and sham inoculated excised hypocotyl segments

<table>
<thead>
<tr>
<th>Time of incubation with [3H]-leucine (5 μCi/ml)</th>
<th>Sham inoculated</th>
<th>Infected</th>
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<tbody>
<tr>
<td>Buffer</td>
<td>Incubated in cycloheximide</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>Buffer</td>
<td>Incubated in cycloheximide</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>I4½ to 15 h</td>
<td>18590</td>
<td>4077</td>
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<tr>
<td></td>
<td>68.1</td>
<td></td>
</tr>
<tr>
<td>I7½ to 18 h</td>
<td>17222</td>
<td>6903</td>
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<tr>
<td></td>
<td>52.9</td>
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</tr>
</tbody>
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(Fig. 4). The accumulation of both virus RNA and virus nucleoprotein was inhibited by exposure to cycloheximide (100 μg/ml) from ½ h after inoculation (not illustrated), but if exposure was delayed until 9 h, infective RNA continued to accumulate, though without the concurrent accumulation of virus nucleoprotein (Fig. 5).

Incorporation of [3H]-leucine into an acid insoluble product

The effect of exposure to cycloheximide (50 μg/ml) on the rate of total protein synthesis was estimated by measuring the rate of incorporation of [3H]-leucine into acid insoluble material. Incubation with the inhibitor from 12 h after inoculation decreased the rates of incorporation by sham inoculated and infected hypocotyls when measured at 15 h and 18 h. At both times inhibition was more marked in sham inoculated than infected hypocotyls. The accumulation of virus nucleoprotein was thus more sensitive to inhibition by cycloheximide than total protein synthesis (Table 1).

DISCUSSION

The infectivity studies show that the multiplication of TNV, like that of tobacco rattle virus, eggplant mosaic virus (Harrison & Crockatt, 1971), TMV (Zaitlin et al. 1968) and Sann hemp mosaic virus (unpublished results, 1971) can be inhibited by cycloheximide but not by chloramphenicol. It is unlikely that chloramphenicol failed to penetrate or was rendered inactive in our experiments, because it had many observable effects, and could be recovered from tissue with full potency. If applied soon after inoculation, cycloheximide (1 to 100 μg/ml) inhibited the accumulation of virus RNA and nucleoprotein with high efficiency, yet at later times incubation with 100 μg/ml failed to inhibit the accumulation of virus RNA, and the same concentrations (1 to 100 μg/ml) were less effective in preventing the accumulation of virus nucleoprotein. Thus it is probable that cycloheximide inhibits the production of at least two proteins necessary for multiplication. Synthesis of the first was extremely sensitive to inhibition by cycloheximide and necessary for the establishment of infection. Synthesis of the second was less sensitive to inhibition by cycloheximide and was essential for the accumulation of virus nucleoprotein, but not for the continued synthesis of virus RNA once an infection had been established. The failure of cycloheximide to inhibit virus RNA synthesis when applied at late times in infection creates a situation which resembles that existing in multiplication of the unstable variants of TNV (Kassanis & Welkie, 1963), in which only virus RNA and not virus nucleoprotein accumulates. For in both instances, once virus RNA has been synthesized, it is relatively long lived and its continued
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synthesis is not suppressed by the failure to form complete nucleoprotein particles. Our
close results are consistent with the contention that the multiplication of TNV is dependent on
proteins synthesized on cytoplasmic but not on plastid ribosomes.

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REFERENCES


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