Changes in the Ribosomes Extracted from Mung Beans Infected with a Strain of Tobacco Mosaic Virus

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SUMMARY

Virus infection causes an increase in the quantity of ribosomes extracted from the hypocotyls of Mung beans. Though this increase is not confined to a particular size of ribosome, presumptive virus messenger RNA is associated predominantly with polyribosomes composed of nine or more monoribosomes.

INTRODUCTION

Tobacco mosaic virus RNA has been used as a messenger in several in vitro studies on protein synthesis (Nirenberg & Matthaei, 1961; Tsugita et al. 1962; Aach et al. 1964) and polyribosome formation (Marcus, Luginbill & Feeley, 1968), but little is known of its association with polyribosomes in vivo. The changes which occurred to the ribosomes of a dark grown plant tissue as a consequence of infection have been investigated.

METHODS

Virus. A virus from cowpea (CMV; Lister & Thresh, 1955) which has been shown to be related serologically to tobacco mosaic virus (TMV) was propagated in the leaves of French beans var. Prince. The virus was purified by differential centrifugation, stored as a 2 % solution and infective RNA prepared from it by treatment with phenol (Schuster, Schramm & Zillig, 1956).

Infectivity assay. The relative infectivity of preparations of virus or virus RNA was determined by inoculating the leaves of tobacco plants (Nicotiana tabacum L. cv. Xanthi) hypersensitive to this virus. The plants were trimmed to three or four leaves by removing the youngest and oldest leaves and each preparation was inoculated the same number of times to all plants and all leaf positions.

Mung beans. Seeds of Phaseolus aureus Roxb. were germinated in the dark in plastic boxes on a filter paper and cotton wool pad wetted with Hoagland’s solution and trace elements (boric acid 0.570 mg./l., manganese chloride 0.314 mg./l., cupric chloride 0.21 mg./l., sodium molybdate 0.204 mg./l., zinc chloride 0.625 mg./l., EDTA ferric monosodium complex 0.720 mg./l.). Fresh humid air was pumped continually through the boxes. After the beans had been germinated for 90 to 100 hr and were approximately 6 cm. long the hypocotyls were inoculated manually with infective RNA in 0.06 M-phosphate buffer at pH 7.0 or with buffer alone.

[32P]orthophosphate incorporation. Segments 6 cm. long were cut from the hypocotyls of Mung beans at various times after inoculation (see text) and 5 g. fresh weight was placed in a

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stopped 3 in. × 1 in. specimen tube with 5 ml. of \(^{32}\)P orthophosphate (activity 600 \(\mu c/ml\)) in 0.01 M-phosphate buffer, pH 6.5. The tube was shaken in a water bath at 28°. After incorporation the tissue was washed five times with distilled water and the ribosomes extracted as below. Determinations of radioactivity were made in a Tracer-Lab Corumatic scintillation spectrometer in a scintillation fluid comprising 5 g. PPO and 0.5 g. dimethyl POPOP in 1 l. toluene.

**Ribosomes, preparation and separation.** Hypocotyl tissue, 5 g., was dropped into liquid nitrogen in a porcelain mortar and pounded for 90 sec. When most of the nitrogen had evaporated, the fragments were ground by hand for 1/2 min. and the fine powder transferred to an ice-cold porcelain mortar. As the tissue began to thaw 3.3 ml. of a medium at pH 8 was added (0.25 M-sucrose, 0.10 M-potassium chloride, 0.01 M-magnesium acetate, 0.005 M-2-mercaptoethanol, 1.0 % sodium deoxycholate, 0.015 M-spermidine and 0.01 M-tris acetic acid buffer). The mixture was ground by hand for 1 min., strained through muslin and centrifuged at 8000 g for 15 min. A sample, usually 4.0 ml., of the supernatant fluid was overlaid on 3.0 ml. of 1.0 M-sucrose in PMMT (0.10 M-potassium chloride, 0.01 M-magnesium acetate, 0.005 M-2-mercaptoethanol, 0.01 M-tris acetic acid buffer pH 8.0) and centrifuged at 105,000 g for 50 min. The pellet was washed and gently resuspended with 1.0 ml. of PMMT and clarified at 6000 g for 3 min. Of this 1.0 ml. was overlaid on a linear gradient of 11 to 37.5 % (w/v) sucrose in PMMT and centrifuged at 65,000 g for 170 min. in the SO 2416 rotor of the M.S.E. Superspeed 40’ ultracentrifuge. The gradient containing the ribosomes was removed by upward displacement from the centrifuge tube and its relative optical density measured at 254 nm. with a Joyce-Loebl Chromoscan densitometer. For determinations of radioactivity, fractions of approximately 0.5 ml. were collected and 100 \(\mu l\) samples counted. When the gradient was to be sampled for infectivity, fractions were collected from the bottom of the tube. All operations were carried out at -1° to +1° and all solutions were made with double-glass distilled water.

**Ribosomes and ribonucleic acids.** The pellet containing the ribosomes from 5 g. of hypocotyl tissue was resuspended in 4 ml. of medium (0.06 M-potassium chloride, 0.01 M-magnesium chloride, 1.0 % sodium lauryl sulphate in 0.01 M-tris + HCl, pH 7.6) and 6.6 ml. of phenol was added to the suspension and the mixture shaken for 15 min. at 0°. The emulsion was centrifuged at 20,000 g for 10 min., the aqueous layer removed and extracted twice more with phenol. Solid potassium acetate was added to adjust to 0.2 M and the nucleic acids precipitated by adding two volumes of cold ethanol. The precipitate was resuspended (0.02 M-sodium acetate, 0.001 M-sodium EDTA, 0.2 % sodium lauryl sulphate, 0.04 M-tris + acetic acid, pH 7.2) and the nucleic acids separated electrophoretically in a 2.6 % acrylamide gel (Loening, 1967). Relative optical density measurements were made with a Joyce-Loebl Chromoscan densitometer. For radioactivity determinations the gels were cut into approximately 100 slices with a Mickle tissue chopper.

**RESULTS**

**Virus multiplication**

The rate of multiplication of CMV in Mung bean hypocotyls varied with temperature (Fig. 1). A temperature of 28° was chosen for incubation in subsequent experiments because it allowed an earlier and higher maximum sap infectivity than at 22° or 35°.

**Ribosome yield and distribution**

Preliminary experiments in which ribosomes were extracted from Mung bean hypocotyls by methods in which the tissue was ground either directly in a buffered sucrose medium
Polyribosomes of infected plants

(Jachmczyk & Cherry, 1968) or in the same medium after freezing in solid carbon dioxide (Lin & Key, 1967) gave poor preservation of polyribosomes. The technique finally adopted (see Methods) yielded amounts of ribosomes similar to those by other methods but gave better preservation of polyribosomes. Higher amounts of monoribosomes and polyribosomes were extracted from CMV-infected Mung beans than from sham inoculated tissues (Fig. 2). There was no indication that polyribosomes of any given size were associated with the RNA of CMV since in infected tissues (Table 1), the content of poly- and monoribosomes increased together (compare Fig. 4, 5).

An isotopic labelling method was therefore used to test the association of polyribosomes with the synthesis of messenger RNA.

![Graph](https://example.com/graph.png)

**Fig. 1.** Infectivity of sap extracted from Mung bean hypocotyls which had been inoculated with CMV-RNA and maintained at 22°, 28°, or 35°. Local lesion numbers were transformed into equivalent concentrations of CMV by interpolation on a dilution curve. △, 35°; ●, 28°; ■, 22°.

**Table 1. Changes in ribosome content during infection**

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Healthy (Sham inoculated)</th>
<th>Infected</th>
<th>Ratio total ribosomes: infected/healthy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Poly-ribosomes</td>
<td>Mono-ribosomes</td>
<td>Poly-ribosomes</td>
</tr>
<tr>
<td>0*</td>
<td>497</td>
<td>629</td>
<td>606</td>
</tr>
<tr>
<td>6</td>
<td>631</td>
<td>787</td>
<td>597</td>
</tr>
<tr>
<td>18</td>
<td>743</td>
<td>488</td>
<td>967</td>
</tr>
<tr>
<td>30</td>
<td>604</td>
<td>555</td>
<td>604</td>
</tr>
<tr>
<td>42</td>
<td>642</td>
<td>564</td>
<td>904</td>
</tr>
</tbody>
</table>

Values in the table are proportional to the area between the optical density trace and the base line. * Untreated.
[\textsuperscript{32}P]orthophosphate incorporation into polyribosomes

To determine how long the tissue should be incubated in radioactive buffer to allow incorporation of the label into messenger RNA but not into ribosomal RNA, tissue previously infected for 21\(\frac{1}{2}\) hr was incubated for 5 to 40 min. with [\textsuperscript{32}P]orthophosphate. The ribosomes were prepared as before, the RNA extracted and separated by acrylamide gel electrophoresis and the activity in different fractions determined. Considerable radioactivity was incorporated into ribosomal RNA by 40 min., but none was detected by 20 min.

Since sufficient radioactivity was incorporated into two major fractions of presumptive messenger RNA in 10 to 20 min. (Fig. 3), an incubation time of 15 min. was accepted for subsequent labelling.

Similar amounts of radioactivity were found in the labelled but unfractionated ribosomes extracted from infected or sham inoculated tissue. In the extracts of infected tissue there was more radioactivity in the polyribosomes composed of five or more ribosomes (Fig. 4), but all classes of ribosomes were similarly labelled (Fig. 5) in extracts of sham inoculated tissues. The results of a single sequential experiment are given in Table 2 and the ‘B/A’ ratios from it. Other experiments are given in Fig. 6.

Experiments were done to check that the \textsuperscript{32}P in the large polyribosomes from infected tissue was incorporated covalently into the nucleic acid and not merely absorbed or linked

Fig. 2. The ratio of total ribosomes extracted from infected plants to that from healthy plants. Units are as described in Table 1. Broken lines connect results obtained on the same batch of tissue. The unbroken curve shows the best-fit, second-order polynomial; the mean error plus twice the standard error of the observations was 0.075.
Fig. 3. Electrophoretic separation in a 2·6 % (w/v) acrylamide gel of the labelled RNA extracted from ribosomes of hypocotyls infected from 21½ hr and incubated for 20 min. with $[^{32}]$Porthophosphate. Marker arrows indicate the positions to which 25, 18 and 5S ribosomal RNA migrated in a replicate gel stained with toluidine blue.

Fig. 4. Separation of ribosomes from tissue which had been infected for 39½ hr and incubated with $[^{32}]$Porthophosphate for 15 min. Optical density, unbroken line; radioactivity, broken line.
in some non-specific way. Ribosomes were extracted from hypocotyl tissue (infected for 24 hr and incubated with $^{32}$P-orthophosphate for 20 min.) and in one experiment 88 counts/sec. were associated with intact ribosomes (separated by centrifugation in a sucrose density gradient) whereas 77 counts/sec. were associated with the nucleic acid extracted from the ribosomes (separated from free nucleotides and small oligonucleotides by electrophoresis in polyacrylamide gels). Thus 87% of the radioactivity incorporated into ribosomes seems to be incorporated into the messenger RNA.

Table 2. Incorporation of $^{32}$P into the RNA associated with ribosomes

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>A. Healthy sham inoculated</th>
<th>B. Infected</th>
<th>Ratio (B)/(A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0*</td>
<td>1.94</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>0</td>
<td>2.11</td>
<td>1.97</td>
<td>0.93</td>
</tr>
<tr>
<td>6</td>
<td>1.93</td>
<td>2.05</td>
<td>1.06</td>
</tr>
<tr>
<td>18</td>
<td>1.55</td>
<td>2.44</td>
<td>1.57</td>
</tr>
<tr>
<td>30</td>
<td>3.80</td>
<td>10.78</td>
<td>2.84</td>
</tr>
<tr>
<td>42</td>
<td>1.62</td>
<td>2.16</td>
<td>1.33</td>
</tr>
</tbody>
</table>

Large polyribosomes are those composed of 5 or more monoribosomes and those composed of 2, 3 or 4 monoribosomes are designated small. Incorporation values and specific activities (counts/sec./unit wt to polyribosomes) in units proportional to the area between the optical density trace and the baseline.

* Untreated.

Fig. 5. Separation of ribosomes obtained from tissue which had been sham inoculated 39½ hr. previously and incubated with $^{32}$P-orthophosphate for 15 min. Optical density, unbroken line; radioactivity, broken line.
Fig. 6. The ratio of specific activities 'B/A' as defined in Table 2. Dotted lines connect results obtained on the same batch of tissue. The unbroken curve shows the best-fit, second-order polynomial; the mean error plus twice the standard error of the observations was ±0.525.

Fig. 7. Infectivity of consecutive 3.5 ml. ribosome fractions separated in a 11 to 37.5 % (w/v) sucrose gradient. The ribosomes were extracted from hypocotyls inoculated 38½ hr previously with CMV-RNA. Areas bounded by broken lines show infectivity before treatment with ribonuclease (25 µg/g.), as are bounded by unbroken lines after treatment. The optical density curve was obtained from another sample of the ribosome preparation separated on a similar gradient at the same time. The marker arrow indicates the position to which whole virus particles migrated when CMV was mixed with ribosomes prior to separation.
Association of infectivity with polyribosomes

Ribosomes of different sizes were obtained from tissue infected for 38.4 hr and then assayed for infectivity either directly or after incubation with ribonuclease 25 µg/ml for 15 min. at 35°C (Fig. 7); higher ribonuclease concentrations in inocula of CMV caused a reduction of infectivity. Treatment with ribonuclease reduced the infectivity of fractions obtained from the bottom of the gradient in this and two other experiments.

DISCUSSION

As the range of sizes of ribosomes is different in similar tissue from different batches of plants or from plants at slightly different times (Loening, 1968), more reliable experiments were made by sequential use of single batches of plants. Also, Mung bean hypocotyls inoculated with CMV–RNA do not show synchronous infection and changes in the ratio of healthy to infected cells are not comparable to those which occur during the cycle of infection within single cells. However, the quantity of ribosomes extracted from infected tissue increased relative to the control, though with little alteration to the ratios of polyribosomes to monoribosomes. A relative increase has been observed by analytical sedimentation in the amount of 83s ribosomes in Chinese cabbage leaves infected with turnip yellow mosaic virus, though the amount of 114s polyribosomes was unaltered (Reid & Matthews, 1966). As it is not yet possible to determine absolutely the quantity of ribosomes within a tissue, virus infection may merely increase the percentage of ribosomes extracted. This is less likely than a real increase because different extraction techniques give similar results for total ribosome content.

Between 18 and 42 hr after inoculation, when the highest proportions of cells were in an exponential phase of virus synthesis, large polyribosomes were rapidly labelled with 32P in vivo. It is possible that the 32P was incorporated into low molecular weight phosphate esters bound to the ribosomes, but this is unlikely for the following reasons. First, it is unlikely that such material would associate only with large polyribosomes and only with those extracted from infected tissue. Secondly, any low molecular weight phosphate esters present after the RNA was extracted from the ribosome preparations would have eluted rapidly from the gel in which the RNA species were separated. Thirdly, polyribosomes extracted from soybean root tissue could be rapidly labelled in vivo with 32P (Lin, Key & Bracker, 1966), [3H]uridine or [3H]adenine (Lin & Key, 1967). The RNA which could be extracted from them had a higher specific activity than that which could be extracted from monoribosomes and had the characteristics of D- or messenger RNA. Fourthly, the presence of deoxycholate during the extraction of ribosomes prevents such fortuitous association (Pennman, Willems & Greenberg, quoted by Loening, 1968, p. 55).

Babos (1969) has reported a species of RNA which could be labelled with [14C]uracil or [32P]orthophosphate and which was associated with the ribosomes of TMV infected tobacco leaves. He concluded that it was associated mainly with monoribosomes, even though the preservation of polyribosomes in his experiments was poor when compared with the previously reported distribution of polyribosomes extracted from the plastids of the same tissue (Chen & Wildman, 1967).

The lower region of sucrose density gradients into which the larger polyribosomes extracted from infected tissue was sedimented contained infective material. This infectivity is unlikely to be caused by contaminating virus particles, as these migrated to the middle of the gradient and their infectivity was resistant to ribonuclease. It is possible that the infective
material was a rapidly sedimenting form of virus RNA, such as a replicase complex, but it is more likely that it was single-stranded virus associated with ribosomes and spermidine and thereby protected against degradation by ribonuclease. If this labelled RNA may be identified as virus messenger RNA, then the number of monoribosomes with which CMV-RNA associates in vivo is similar to that which TMV-RNA binds in vitro (Marcus et al. 1968).

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REFERENCES


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