POLYRIBOSOME FORMATION AND RNA SYNTHESIS DURING AGING OF CARROT-ROOT TISSUE

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When thin slices are excised from intact storage organs (roots and tubers) of plants, a rapid and dramatic change in the metabolic activity of the tissue ensues. The most obvious measure of this metabolic change is a time-dependent increase in respiration, which differs qualitatively from the basal respiration of the intact tissue.\(^1\)\(^-\)\(^3\) Invertase activity is absent from mature storage tissue but rapidly becomes apparent in washed aerated discs of sugar beet, artichoke, and carrot after a lag of some six to eight hours.\(^4\)\(^,\)\(^5\) The development of invertase activity is an expression of a more general phenomenon since at least four other enzyme activities increased in a similar manner, although these activities were detectable in fresh discs.\(^6\) The increase in enzyme activities was associated with a net increase in total protein.

The nucleic acid content of discs aged in aerated distilled water increased by about 50 per cent during the first 24 hours.\(^7\) The lag in the onset of net nucleic acid synthesis was shorter than that of enzyme activities. Methylated albumin-kieselguhr column and sucrose density gradient fractionation of nucleic acids isolated from discs aged for increasing periods of time indicated that the major components of RNA increased at about the same rate.\(^7\)

Click and Hackett,\(^8\) using potato discs, showed that respiratory changes were dependent upon the synthesis of new RNA and protein since they were inhibited by actinomycin D and puromycin, specific inhibitors of DNA-dependent RNA synthesis and protein synthesis, respectively.\(^9\)\(^,\)\(^10\) Further, the apparent induction and synthesis of invertase and the increase in activity of other enzymes in artichoke and carrot were blocked by a wide range of RNA and protein synthesis inhibitors.\(^5\)\(^,\)\(^6\)

Our results show a marked and rapid association of monoribosomes into polyribosomes in discs of carrot root following excision. RNA synthesis was essential to this transition, and more specifically, a fraction having the properties of messenger RNA was required. These results are discussed in relation to the underlying mechanism of the “activated” metabolism of the discs. This transition probably represents the earliest reported biochemical change resulting from the slicing of these tissues.

Materials and Methods.—Preparation and aging of tissue: Carrots were purchased locally in large batches and stored at 2–4°C. Discs (1 cm diam. and ca. 1 mm thick) were cut from the outer phloem parenchyma region of the carrot root and washed in ice-cold distilled water. They were frozen immediately on dry ice (fresh tissue discs) or transferred to flasks containing distilled water (5 gm fresh weight tissue/50 ml) maintained at 30°C with continuous shaking. All experiments were conducted with and without chloramphenicol at 50 μg/ml. Chloramphenicol inhibited bacterial growth to insignificant numbers without affecting a range of metabolic activities studied in storage tissues during the washing process.\(^11\) Discs which were shaken in distilled water containing 50 μg/ml chloramphenicol for various periods of time will be referred to as “aged discs.”

Treatment with inhibitors and incorporation of radioactive RNA precursors: Discs were blotted dry and placed in a standard volume (5 gm fresh weight tissue/50 ml) of solution and vacuum
infiltrated for 1 min. This facilitated the uptake of the inhibitors and RNA precursors and had no effect on a variety of physiological processes studied in this system. H²-adenosine and P³₂-
orthophosphate were added to the incubation solutions at the concentrations and times indicated
in the figure legends.

Isolation and characterization of ribosomes: Ribosomes were extracted using the method of Lin
et al.¹² with minor modifications. Discs were frozen on dry ice and ground in dry ice by a mortar
and pestle. The powder was then gently homogenized (usually four strokes with eight to ten
quarter turns each) in a 0.25 M sucrose solution containing 0.05 M tris buffer (pH 7.4), 0.015 M
KCl, 0.02 M MgCl₂ and 0.001 M Cleland's reagent. The homogenate was filtered through two
thicknesses of Miracloth and the filtrate centrifuged at 20,000 × g for 20 min. Ribosomes were
prepared from the supernatant solution by layering the sample (5.5 ml) over successive layers of
0.5 M (3.0 ml) and 1.6 M (3.5 ml) sucrose (each contained 0.05 M tris (pH 7.4), 0.015 KCl, and
0.005 M MgCl₂) followed by centrifugation at 105,000 × g for 4 hr at −12°.¹³ The ribosome
pellet was suspended in 0.05 M tris (pH 7.4) containing 0.015 M KCl and 0.005 M MgCl₂. All steps
were carried out at about 0°.

The ribosome preparations were either used at this stage in studies of in vitro amino acid incor-
poration following the methods of Mans and Novelli¹⁴ and Williams and Novelli,²⁰ or characterized
as follows: A 0.7-ml sample containing about 1 mg of ribosomes was layered onto a linear sucrose
gradient (10-34%) and centrifuged at 24,000 rpm in the SW 25 Spinco rotor for 2 hr at −12°.
The distribution of ribosomes in the gradients was determined by collection with continuous re-
cording in an ISCO model D density gradient fractionator. Collection was made from the top
of the gradient at a syringe speed of 3¹/₂ ml/min with the OD profile being made at 254 μμ using
a l-cm light path. When ribosomes were isolated from tissue which had been labeled with H²-
adenosine, 0.5-ml fractions were collected, made to 2 ml, and the OD was measured at 260 μμ.
The samples were precipitated with an equal volume of 10% trichloroacetic acid (TCA), collected
on nitrocellulose membrane filters (type B-6, S and S), washed successively with 5 and 0.5% TCA,
and dried before counting in a Packard liquid scintillation spectrometer.

Nucleic acids were extracted from the tissue by a phenol method¹⁶ and fractionated on methyl-
ated albumin-kieselguhr columns (MAK) described by Mandell and Hershey.¹⁷ The NaCl
elution gradients are given in the figure legends.

Radioisotopes were obtained from Schwarz BioResearch; and polyuridylic acid, ATP, GTP,
phosphoenolpyruvate, and pyruvic kinase from Calbiochem. Actinomycin D was a gift from
Merck, Sharp and Dohme, Rahway, N.J., and 5-fluorouracil a gift from Hoffman-La Roche and
Company, Nutley, N.J.

Results.—Polyribosome formation during aging: Ribosomes from freshly cut and
six-hour-aged discs were fractionated on 10-34 per cent linear sucrose gradients
(Fig. 1). The ribosome preparations from fresh discs consisted of about 90 per
cent monoribosomes and 10 per cent polyribosomes, whereas the proportion of
polyribosomes increased to about 60 to 65 per cent in discs aged for six hours. The
time course of the monoribosome to polyribosome transition is shown in Figure 2.
There was no marked lag associated with the polyribosome formation, with the
maximum rate occurring during the first hour after excision. The proportion of
ribosomes present as polyribosomes did not change appreciably after six hours.

Treatment of fresh discs with actinomycin D greatly depressed the formation of
polyribosomes during aging (Table 1, Fig. 3). During the first hour of aging, the
transition from monoribosomes to polyribosomes was inhibited by about 65 per
cent. There was no net change in the proportion of polyribosomes subsequent to
the first hour in the actinomycin D-treated tissue. 5-Fluorouracil (FU) did not
appreciably alter the proportion of ribosomes present in the polyribosome structure
during six hours of aging (Table 1, Fig. 3). Taken together these results indicated
that RNA synthesis was essential for polyribosome formation during excised in-
cubation. Since FU selectively inhibits RNA synthesis in plant tissues,¹⁸⁻¹⁹ the
failure of FU to inhibit the monoribosome to polyribosome transition suggested
that the synthesis of all classes of cellular RNA was not essential for polyribosome formation.

RNA synthesis during aging: Figure 3 compares sucrose gradient profiles of ribosomes isolated from tissue which had been incubated for six hours with the addition of H\textsuperscript{3}-adenosine to the medium during the terminal 30 minutes (5\textsuperscript{1/2} to 6 hours). There was marked incorporation of precursor into the RNA of ribosomes of control tissue, with the polyribosome region of the gradient showing a higher specific activity than the monoribosome region. FU depressed incorporation of H\textsuperscript{3}-adenosine, with the inhibition being greater in the monoribosome fraction. Actinomycin D strongly inhibited incorporation of H\textsuperscript{3}-adenosine into both the monoribosome and polyribosome regions of the gradient. Table 1 summarizes the effects of actinomycin D and FU on H\textsuperscript{3}-adenosine incorporation into the monoribosomes and polyribosomes during the terminal 30 minutes of a one-hour and a six-hour incubation. During the first 30-minute labeling period (30 minutes to 1 hour), the polyribosomes attained about a five-fold higher specific activity than the monoribosome fraction in both control and FU-treated tissue. As Figure 3 and Table 1 indicate, the use of FU resulted in a greater differential labeling of the poly-
TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Exposure to H$_3$-adenosine (hr)</th>
<th>Polyribosomes (%)</th>
<th>Specific Activity (cpm/OD 260)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5–1.0</td>
<td>45</td>
<td>650</td>
</tr>
<tr>
<td></td>
<td>5.5–6.0</td>
<td>62</td>
<td>11,360</td>
</tr>
<tr>
<td>Actinomycin D (20 µg/ml)</td>
<td>0.5–1.0</td>
<td>23</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>5.5–6.0</td>
<td>22</td>
<td>400</td>
</tr>
<tr>
<td>5-Fluorouracil (2.5 × 10$^{-3}$M)</td>
<td>0.5–1.0</td>
<td>44</td>
<td>620</td>
</tr>
<tr>
<td></td>
<td>5.5–6.0</td>
<td>59</td>
<td>4,980</td>
</tr>
</tbody>
</table>

Discs were aged for 1 or 6 hr, and 250 µc H$_3$-adenosine (6.0 c/mmole) added during the final 30 min of each period. The % polyribosomes of fresh discs was 10% in these experiments.

ribozymes up to six hours than was the case in control tissue, although incorporation of H$_3$-adenosine was inhibited between 5½ and 6 hours. Actinomycin D inhibited H$_3$-adenosine incorporation by about 70 per cent during the 30-minute to 1-hour interval and greater than 90 per cent during the 5½– to 6-hour period, paralleling the inhibition of polyribosome formation.

To ascertain if FU selectively inhibited ribosomal and soluble RNA synthesis in the carrot discs without appreciably altering the synthesis of DNA-like RNA, or D-RNA (a fraction of RNA having properties of messenger RNA$^{18, 19}$), discs of carrot were aged in distilled water with and without FU for four hours followed by a two-hour incubation in P$_{32}$-orthophosphate. RNA was isolated from the control and FU-treated tissues and subsequently fractionated on MAK columns (Fig. 4). Clearly, FU almost completely inhibited the incorporation of P$_{32}$ into ribosomal RNA and into the DNA. There was considerable inhibition of incorporation into the two sRNA fractions although not into the low-molecular-weight RNA (P$_{32}$ profile) eluting between the double peak OD profile. There was only slight inhibition by FU of P$_{32}$-incorporation into D-RNA.

In vitro amino acid incorporation by ribosome preparations: The ability of ribosome preparations from freshly cut and six-hour-aged discs to incorporate C$^{14}$-leucine is shown in Figure 5. Clearly, the ribosomes from aged discs (about 65% polyribosomes) were substantially more active in C$^{14}$-leucine incorporation than ribosomes from fresh discs (about 10% polyribosomes). The ability of ribosomes from discs aged for increasing intervals to incorporate C$^{14}$-leucine (10-minute incorporation period) is shown in Figure 6. There was a close parallel between the level of C$^{14}$-leucine incorporation and the proportion of polyribosomes in each preparation (see Fig. 2).

The possibility remained that although the inclusion of FU in the aging medium did not significantly affect the monoribosome to polyribosome transition, polyribosomes formed might be inactive in in vitro amino acid incorporation. Accordingly, discs were aged for six hours in FU, actinomycin D, and water. The ribosomes were isolated, and the in vitro amino acid-incorporating activities compared (Fig. 7). FU marginally inhibited in vitro incorporation of C$^{14}$-leucine compared with the water control, while actinomycin D reduced incorporation by some 55 per cent.

To determine if ribosomes from fresh discs were biologically active, i.e., capable of binding synthetic messenger RNA and directing amino acid incorporation, relative to ribosomes from aged discs, the incorporation of C$^{14}$-phenylalanine with and
without synthetic polyuridylic acid (poly U) was measured. Without poly U, ribosomes from six-hour-aged discs were more active in C\textsuperscript{14}-phenylalanine incorporation than ribosomes from fresh discs (Table 2). However, in the presence of poly U, there was only a slight difference in incorporation of C\textsuperscript{14}-phenylalanine by the ribosome preparations.

Discussion.—Our data show that polyribosomes rapidly form during the initial phases of aging of discs of the carrot root. The proportion of polyribosomes in the ribosome preparations increased from about 10 per cent in fresh discs to about 45 per cent after one hour of aging and reached a constant value of about 65 per cent

<table>
<thead>
<tr>
<th>Source of ribosomes</th>
<th>Additions</th>
<th>C\textsuperscript{14}-phenylalanine incorporation (cpm/mg ribosomal RNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh tissue</td>
<td>None</td>
<td>2,030</td>
</tr>
<tr>
<td>Poly U</td>
<td>60,430</td>
<td></td>
</tr>
<tr>
<td>Six-hour-aged tissue</td>
<td>None</td>
<td>3,311</td>
</tr>
<tr>
<td>Poly U</td>
<td>65,793</td>
<td></td>
</tr>
</tbody>
</table>

The incubation mixture was identical to that in Fig. 5 except 0.5 μc C\textsuperscript{14}-phenylalanine (350 μc/μmole) was added in place of C\textsuperscript{14}-leucine and the MgCl\textsubscript{2} concentration was raised to 10 μmole per incubation mixture. Poly U was used at 100 μg per incubation mixture. Incubations were terminated after 30 min at 37°C.
after six hours. This transition from monoribosomes to polyribosomes appears to be one of the earliest biochemical changes associated with aging of discs from storage tissues of plants, and a change which probably underlies the onset of protein (enzyme) synthesis and the activated metabolism of aged discs.

The formation of polyribosomes is dependent upon RNA synthesis. Polyribosome formation was inhibited about 65 per cent by actinomycin D during the first hour of aging and almost completely thereafter. This inhibition of polyribosome formation was paralleled by the inhibition of total RNA synthesis (H\textsuperscript{3}-adenosine incorporation into RNA) by the antibiotic. 5-Fluorouracil, while not seriously affecting polyribosome formation, did inhibit ribosomal RNA synthesis almost completely and to a lesser extent soluble RNA synthesis. As reported for other plant tissues,\textsuperscript{18, 19} the synthesis in carrot discs of D-RNA, and a low-molecular-weight RNA, eluting in the region of soluble RNA, was not appreciably affected by FU. These results suggest that the synthesis of D-RNA, which is presumably messenger RNA,\textsuperscript{12, 18, 20} is essential for polyribosome formation during the early aging process. These observations are consistent with and extend the earlier results which showed a requirement for RNA and protein synthesis for the enhanced respiratory activity of potato discs\textsuperscript{8} and the increase in activity of invertase and other enzymes during aging of artichoke and carrot discs.\textsuperscript{5-7}

The increase in the proportion of polyribosome-associated ribosomes was closely paralleled by an increase in the \textit{in vitro} incorporation of amino acids by the ribosome preparations. Ribosomes from six-hour-aged discs (about 65% polyribosomes) incorporated five to six times more C\textsuperscript{14}-leucine than ribosomes from fresh discs.
(about 10% polyribosomes). Ribosomes from FU-treated discs (about 60% polyribosomes) incorporated C14-leucine to about the same extent as ribosomes from control discs. Treatment of discs with actinomycin D resulted in a marked decrease in both the in vitro incorporation of C14-leucine by isolated ribosomes and the proportion of polyribosomes. The poly U-directed incorporation of C14-phenylalanine by ribosome preparations from fresh and aged discs was about the same. Thus the low level of polyribosomes in fresh discs was apparently unrelated to an inability of ribosomes to bind messenger RNA and initiate protein synthesis.

Possibly the simplest interpretation of the results is that the availability of messenger RNA limits the proportion of ribosomes present as polyribosomes in fresh discs. Upon excision, an enhanced synthesis of messenger RNA occurs with a resulting increase in polyribosomes. Conceivably another component, such as an enzyme or a minor species of sRNA essential to protein synthesis and thus polyribosome formation and function, could be limiting in the intact tissue. Our results do show, however, that RNA synthesis (presumably messenger RNA) is essential to allow polyribosome formation.

**Summary.**—The proportion of ribosomes present as polyribosomes rapidly increases from about 10 per cent in fresh discs of carrot root to a constant value of about 65 per cent during aging of discs. The results based on the effects of actinomycin D and 5-fluorouracil indicate that the synthesis of a fraction of RNA having the properties of messenger RNA is required for this transition. The rapid increase in polyribosomes is one of the earliest biochemical changes observed during aging of storage-tissue discs.

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