CHAPTER 12

The Occurrence and Properties of Polysomes in Plant Tissues

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The concept of the polysome as an aggregate of ribosomes joined by messenger RNA arose from the necessity of accommodating the long messenger molecule on a ribosome and allowing the ribosome to translate the message into the polypeptide chain (Wettstein *et al.*, 1963; Rich *et al.*, 1963). The existence and functioning of polysomes has been well established in animal tissues; the question here is to determine whether polysomes can be similarly found in plants and what techniques can be used to isolate them.

I. General Properties of Polysomes

Strings or clumps of ribosomes can be seen, with the use of the electron microscope, both in whole cells and in cell fractions from homogenates. The isolated polysomes sediment on centrifugation more rapidly than the single ribosomes, in a series of peaks according to the number of ribosomes per polysome. It would be expected that the longer the polypeptide chain being synthesized, the longer is the messenger RNA coding for it and therefore the greater the number of ribosomes in one polysome. Very frequently, the polysomes are attached to endoplasmic reticulum membranes in the cell and this is particularly the case in the more active protein synthesizing tissues such as pancreas and developing plant cells. Similar membrane-bound

polysomes have also been found in bacteria, for example in *B. megaterium* by Schlessinger (1963).

Descriptions of polysomes *in situ* in fixed tissues were in fact published several years before their significance was appreciated. The occurrence of rapidly sedimenting ribosomes had also been noted earlier but in most cases these results were ascribed to nonspecific aggregation and remained unpublished. Among the early findings, Watson (1959) had described coils of ribosomes in rat liver, attached to the endoplasmic reticulum or to the nuclear membrane. This was even before messenger RNA had been described and a true interpretation of the pictures could therefore not be given. In these pictures, it appears that the larger ribosomal subunit is to the outside of the coil and the smaller to the inside. More recently, Sabatani *et al.* (1966) suggest, in a biochemical and electron microscopic study of membrane-bound ribosomes, that the larger subunit is the one that is attached to the membrane. The smaller subunit can be dissociated from the whole microsome with EDTA leaving the larger one still attached. The relationship of these biochemical results to the electron microscope studies of the polysome coils remains obscure and requires further study; the nature of the attachment of the coils and whether the coiled arrangement is a property of the membrane or of the messenger connecting the ribosomes is not known.

The structure of isolated polysomes has been investigated with the electron microscope using shadowing, negative staining or positive staining. In the latter, when the ribosomes have been stretched apart from each other, the strand of messenger RNA can be seen (Dass and Bayley, 1965; Slayter *et al.*, 1963). The pictures from Das and Bayley show, apparently, branched messenger RNA in the form of Y rather than X. While it is possible that the polysomes can become entangled during isolation, this is not so likely to lead to the Y type of branch. It is probable therefore that some ribosome interactions causing clumping or stringing are nonspecific and not due to the messenger RNA. Some of the strands which are visible could be dried stain (G. Palade, personal communication).

While there are inconsistencies, as indicated above, in our understanding, we can consider the following five criteria to be characteristic of polysomes:

(1) The polysomes should become labelled with nascent protein after brief *in vivo* incorporation of labelled amino acids by the tissue; i.e., it must be shown that the polysome is the active structure in protein synthesis.

(2) The isolated polysomes should be capable of amino acid incorporation into protein *in vitro*, more so than the free single ribosomes.

(3) Messenger RNA must be a part of the polysome structure.

(4) There should be some correlation between the yield or content of polysomes in the tissue and the protein synthesizing activity of the tissue.

(5) The isolated polysome structure should be fragile to ribonuclease action which will degrade the messenger string and release free ribosomes; similarly shearing forces may break the polysome structure.

We consider below how such structures can be identified and isolated from plant tissues and some of their properties.

II. Detection of Polysomes *In Situ*

There are now many published pictures of fixed plant cells in which the ribosomes occur in clumps or coils. One example can be taken from studies by Bonnet and Newcomb (1965) with radish in which the endoplasmic reticulum is in places dilated to a vesicular structure. In this case one can see the ribosome-covered, "rough" endoplasmic reticulum in every gradation from transverse to tangential section. This gives an opportunity to show that the bulk of the membrane attached ribosomes occur in coiled arrangements containing 10 to 40 ribosomes. Helical arrangements of ribosomes, probably not attached to membranes, have also been observed (Echlin, 1965). In this laboratory, we have found the coiled and helical forms within the same cell in cultured explants of Helianthus tuber (J. Tullet, unpublished). These studies have not demonstrated that the coiled arrays are in fact polysomes in the sense that the ribosomes are necessarily bound to each other by messenger RNA. The cytological study must now be combined with a biochemical one.

III. Methods of Isolation of Polysomes

A. Homogenization of the Tissue

In plant cells the tough cell wall leads to homogenization problems which have already been mentioned in this symposium; it is difficult to break the cell wall without disrupting, to some extent, the polysome structure. A number of methods have been tried from mechanical chopping with razor blades (Spencer and Wildman, 1964; used for chloroplasts) to grinding with mortar and pestle or homogenizing gently in teflon-glass homogenizers with carefully controlled clearances. Another technique is to freeze the tissue in dry ice or liquid nitrogen and grind the frozen tissue to a powder; the powder is then broken up into medium in a teflon homogenizer (Lin and Key, 1967). None of these methods is entirely satisfactory and others will be needed.

The homogenizing medium must be constituted so as to stabilize the polysomes as well as other structures which could release ribonucleases. It must contain magnesium ion to maintain the integrity of the ribosomes and sodium or potassium ion or both. The ratio of magnesium to monovalent salts is probably more critical than the absolute concentration (Breillart and Dickman, 1966). Sucrose may be added to preserve other cell particles. The

medium will be buffered, usually with Tris, and close control of the pH may be necessary especially when using deoxycholate to dissolve the endoplasmic reticulum membranes (Waters and Dure, 1966). Such detergents should, of course, be added only after the larger cell particles have been removed by centrifugation; it is probably difficult, however, to spin down mitochondria and lysosomes completely without also sedimenting some of the larger membrane-attached polysomal particles.

Throughout these procedures, some nuclease inhibitor will probably be required. There is a range of different nucleases in most plant cells and one cannot expect to find an inhibitor which inhibits all of them and does not interfere with the fractionation procedures. The inhibitor must be chosen to suit the particular tissue. Bentonite is one of the most commonly used nuclease inhibitors; this is a negatively charged earth which binds the positively charged basic proteins, effectively removing them from solution. The method of purification of bentonite described by Singer and Fraenkel-Conrat (1961) for the preparation of Tobacco Mosaic virus RNA involves washing the bentonite with EDTA and then with water or salts. R. Watts (unpublished results) has shown that the amount of EDTA remaining in the gelatinous precipitate is large and would bind much of the magnesium ion added to the homogenizing medium. The washing procedure must therefore be repeated many times. Further, because of its negative charge, bentonite also has a tendency to bind ribosomes and polysomes (Tester and Dure, 1966); this is particularly the case with magnesium rather than sodium bentonite, so much so that magnesium bentonite has been used by Dunn (1965) to remove ribosomal materials from leaf homogenates and to extract t-RNA from the supernatant. There is considerable discrepancy among different workers on the effects of bentonite. We have found in this laboratory that even sodium bentonite binds ribosomes and t-RNA (P. Babos and J. Ingle, unpublished results) whereas Dr. R.Watts considers that only damaged ribosomes attach to bentonite. A quantitative check of yields is advised (Tester and Dure, 1966).

Polyvinyl sulphate has been used as an RNase inhibitor in a number of cases, such as in one of the earliest extractions of polysomes from plant tissues by Clark *et al.* (1964). They pointed out that it is satisfactory to add PVS in low concentration to the homogenate (Chinese cabbage leaf) in which the whole cell sap is present but that if it is used at a later stage in the preparation for resuspending the polysomal pellet, it causes degradation of the polysomes. This phenomenon has since been studied in another connection by Vanyushin and Dunn (1966) who showed that PVS reversibly dissociates ribosomes to their subunits. The degradation noted by Clark and his colleagues is likely, therefore, to have been a dissociation rather than degradation and the cell sap evidently contained something, such as other basic proteins, which prevented this dissociation.

Magnesium ion itself is an inhibitor of some ribonucleases and some media used have contained no other inhibitor (Lin and Key, 1967). As mentioned above, the magnesium concentration used in relation to that of sodium may be critical (Breillart and Dickman, 1966). Zinc ion is also a strong inhibitor of some plant nucleases in low concentration (Stockse and Vandendriessche, 1961) and was used by Barker and Rieber (1967) in the isolation of polysomes from germinating pea seeds. They used magnesium and zinc together and found that the zinc concentration was critical; 1 mM Zn^{2+} failed to inhibit the nucleases and 10 mM caused aggregation. They therefore used 5 mM Zn^{2+}.

B. ANALYSIS OF POLYSOMES BY CENTRIFUGATION

Two procedures of density gradient centrifugation have been used. The cell homogenate is first cleared by a medium speed centrifugation (up to 20,000 $\times$ *g*) and the supernatant is then either layered immediately on the density gradient or centrifuged to pellet the polysomal fraction, perhaps through sucrose layers as described by Wettstein *et al.* (1963). The pellet is resuspended and analysed on a sucrose gradient. The former method has the advantage of speed, minimizing the time of nuclease action, but suffers because frequently not sufficient of the dilute homogenate can be layered on the gradient and uv absorbing materials frequently interfere with the final scanning of the gradient. In the latter method, the polysomes are concentrated and purified but more time is allowed for nuclease action and there may be considerable breakdown in resuspending the pellet by shearing action. Analytical centrifugation in the Spinco model E has also been used with schlieren optics. A high concentration of polysomes is required to give the schlieren pattern but uv absorbing materials do not, of course, interfere.

IV. Properties of Plant Polysomes

A. EXAMPLES FROM SEVERAL SPECIES

Phillips *et al.* (1964) described the density gradient analysis of polysomes from slime moulds. These show the monomer ribosome and the expected regular series of peaks of increasing sedimentation constant. The bulk of the ribosome content of the homogenate was present as polysomes and the pentamer peak was the most frequent of these. They found that short incubation *in vivo* of the mould in labelled leucine resulted in preferential labelling of the polysomes over the free ribosomes and ribonuclease treatment caused the polysomes to break down leaving only the "monosomes". The term "monosome" is conveniently used to refer to such ribosomes as are obtained by breakdown of polysomes by ribonuclease action or other means such as shearing. The term ribosome is then confined to the naturally occur-

ring monomer; its properties are usually different from the monosome since it does not contain fragments of broken messenger RNA nor of nascent protein.

Clark *et al.* (1964) used analytical centrifugation to demonstrate the presence of polysomes in Chinese cabbage leaves. Small amounts of polysomes were seen in the schlieren pictures up to the pentamer. Again low concentrations of ribonuclease caused degradation of the polysomes. That the concentration of ribonuclease may be important if it is to be used diagnostically for polysomes was suggested by the work of Henney and Stork (1964) on *Neurospora* polysomes. Their schlieren patterns show the ribosomal subunits, the 80S monomer and a number of polysome sizes. Ribonuclease, at a concentration of 1 μg/ml, degraded the polysomes to monosomes as expected but 25 μg/ml caused aggregation of the ribosomes and gave spurious patterns.

Watts and Mathias (1965 and unpublished results) extracted polysomes from germinating barley seeds. In the absence of bentonite, polysomes were obtained in low yield and were unstable; extended centrifugation caused breakdown. In the presence of bentonite, prepared by extensive washing as mentioned above, the polysomes accounted for 70 to 80% of the total ribosome population and the authors consider this ratio to be closely similar to that *in vivo*. These preparations could be incubated at 37° for 5 min with very little breakdown. Incubation with ribonuclease 10^{-3} or 10^{-1} μg/ml resulted in a considerable breakdown of the polysomes as shown by an increase in the proportions of the monomer and smaller polysomes. The degradation was not complete, however, and the density gradient patterns were still similar to many normal polysome extracts described in the literature. The concentrations of ribonuclease used were 10 and 1000 times smaller than those usually employed for this purpose and the experiments show the extreme sensitivity of polysomes to nuclease action. In the presence of bentonite, however, these polysome preparations were stable at 5° for several days.

Lin *et al.* (1966) have described the isolation of polysomes from soya bean roots using the technique of grinding the frozen tissue followed by gentle homogenization in a medium containing Tris, KCL and Mg^{2+} ion but no other inhibitor of nucleases. No deocycholate was used since the bulk of the ribosomes of this tissue are free of membranes. They were able to show, by labelling *in vivo* with ^{32}P or with labelled adenosine and uridine, that isotope was very rapidly incorporated into these polysomes and that the base composition of the labelled RNA was similar to the "D-RNA" which is considered to be messenger RNA. The specific activity of RNA prepared from the polysomes was higher than that from the ribosomes. The density gradient patterus did not show as high a proportion of the larger polysomes as those

of Watts and Mathias (1965) but they were perfectly adequate to show that the D-RNA was specifically associated with the polysomes. Electron microscope pictures of negatively stained preparations of these polysomes showed that they occur in clumps whereas after nuclease treatment they appear singly or in dimers. Lin *et al.* (1966) further showed that the polysomes were several times more active in incorporating leucine or phenylalanine into protein *in vitro* than were the free ribosomes or preparations which had been degraded into monosomes.

The above experiments are examples which demonstrate that the polysomes isolated from plants do have the characteristics outlined in the five points above.

B. THE EFFECTS OF INHIBITION OF PROTEIN SYNTHESIS

The influence of the physiological state of the tissue on the properties and content of polysomes can now be considered; first the influence of metabolic inhibitors and conditions of shock and then changes during growth and cell development.

Several experiments with plants and animals have shown that the extent to which the ribosomes occur as polysomes varies continuously according to the physiological state of the cell. Clark *et al.* (1964) found that they were unable to extract polysomes from Chinese cabbage leaves after even momentary wilting. Similarly, Lin and Key (1966) found that anaerobiosis caused the complete disappearance of polysomes from soya bean roots. Merely harvesting the tissue from the growing medium and incubation in aerated water with shaking resulted in some loss. It is probable that the shock of the move caused a large loss of polysomes and that the proportion found after 1 hr shaking in water represents a late stage in recovery. When the roots were incubated in still water, there was a greater loss than in stirred water. Bubbling nitrogen, or incubation in DNP, resulted in a complete loss. This suggests that energy is continuously required for the maintenance of the polysome population. On the other hand, incubation for 1 hr in actinomycin D, which was shown to inhibit RNA synthesis almost completely, had little effect on the proportion of polysomes. In all these conditions, the activity of the isolated ribosome preparations in the incorporation of leucine *in vitro* was directly related to the proportion of polysomes. Waters and Dure (1966) similarly showed that during the first 16 hr germination of cotton seeds, actinomycin D almost entirely inhibited the synthesis of RNA (measured by ^{32}P incorporation) but the proportion of polysomes was maintained, protein synthesis continued and the germination of the seed at this stage was not affected. It is clear that continuous synthesis of RNA is not necessarily required for the maintenance of polysomes and we can conclude that these tissues contain a high proportion of stable, active messenger RNA. The

immediate control of the amount of polysomes and of protein synthesis seems to depend more on the state of the tissue, including the degree of shock provided by the experimentalist, than on the short-term rate of RNA synthesis.

The recovery from shock provides some clues concerning the fate of the messenger RNA in the absence of polysomes. Lin and Key (1967) followed the time course of recovery of the soya bean root during aeration after anoxia. At 30 min after the start of aeration, a proportion of polysomes had reformed; thereafter the recovery continued more slowly. The rapid recovery over the first 30 min was only slightly inhibited by actinomycin D, while the continued slower recovery was strongly inhibited. There are clearly two phases in the recovery, the first of which is independent of RNA synthesis. It appears, therefore, that the loss of polysomes due to anoxia does not destroy all of the messenger RNA but dissociates it from the ribosome. A proportion of the messenger RNA survives in the cytoplasm and can be used for the formation of polysomes as soon as energy is again provided.

Similar conclusions were reached by Conconi *et al.* (1966) with reticulocyte cells synthesizing haemoglobin. They found that fluoride ion rapidly resulted in a loss of polysomes but that protein synthesis recommenced and polysomes were formed on the removal of the fluoride. This was in the absence of RNA synthesis since these cells have no nuclei. Ninety per cent of the protein synthetic activity was recovered when only 10 to 20% of the polysomes had re-formed. In this case, therefore, the proportion of polysomes was not directly related to the rate of protein synthesis. The limiting factor appeared to be the supply of some form of energy for the peptide bond formation; faster chain growth on a smaller number of polysomes led to similar rates of protein synthesis.

These findings taken together suggest that the breakdown of polysomes under anoxic or other unfavourable conditions is due to the inhibition of the attachment of ribosomes to messenger RNA and perhaps to reduction of the initial rate of travel of ribosomes along the messenger but that those polypeptide chains which were in the course of being synthesized are completed. Thus ribosomes leave the polysomes at the end of the read-out process but under unfavourable conditions chain initiation is inhibited and polysomes disappear. It is also seen that messenger RNA can exist in stable form in the cytoplasm free of ribosomes. This interpretation predicts that the ribosomes formed under anoxic conditions are free of nascent protein and of messenger fragments. Lin and Key (1967) showed that after incubation *in vivo* with radioactive leucine, the ribosomes which accumulate during anoxic conditions are not labelled. This is in contrast to the monosomes which are formed by degradation of polysomes by nucleases. One can also predict that if protein synthesis is inhibited by a substance which prevents

the read-out process, the polysomes should remain intact under anoxic conditions. Cycloheximide is an inhibitor of this type (Wettstein *et al.* 1964) in contrast to puromycin which acts as an analogue of t-RNA. Lin and Key found that, in fact, in the presence of cycloheximide, polysomes were maintained under anoxic conditions for at least 2 hr and protein synthesis was inhibited. On the other hand, Waters and Dure (1966) found that 16 hr treatment of the cotton seeds with cycloheximide inhibited both protein and RNA synthesis and the polysomes were lost. However, this treatment is very much longer than the experiments on the soya beans and other physiological factors presumably become important.

These experiments show that the presence of messenger RNA in the cytoplasm does not necessarily result in the formation of polysomes; the messenger can exist in the cytoplasm free of ribosomes. There is thus some precedent for the possibility that protein synthesis can be controlled at the level of translation in the cytoplasm by metabolic conditions. It becomes of interest therefore to enquire whether in other systems in which protein synthesis is controlled the mechanism is through the control of messenger RNA synthesis or at translation.

C. THE CONTROL OF POLYSOMES DURING GROWTH

A few changes in the levels of polysomes in tissues under different conditions of growth or metabolism have been observed. These must be regarded as preliminary experiments in the search for suitable systems in which the genetic or translational control of protein synthesis can be studied.

Thus Clark *et al.* (1964) in their experiments with Chinese cabbage leaves noted that there was a diurnal variation in the amount of polysomes obtained. In the dark, polysomes were gradually lost and light stimulated a production of polysomes within 30 min. This required RNA synthesis so that it would appear that at least a part of this control is at the genetic level.

The onset of protein synthesis during the germination of seeds or spores is being investigated in a number of laboratories.

Henney and Stork (1964) found that the ascospores of *Neurospora* did not contain polysomes but that polysomes are formed over several hours during germination. Growing tissue lost its polysomes during periods of starvation.

In the seeds of higher plants there is considerable controversy about whether polysomes exist in the dry seed, at what stage protein synthesis begins and whether RNA synthesis is necessary for this. Waters and Dure (1966) suggested that in cotton seed, polysomes are present throughout and that while RNA synthesis can be detected during the first 16 hr of germination, it was not essential for the maintenance of the polysomes, for protein synthesis or for the immediate process of germination. The RNA synthesis could be inhibited with actinomycin D without inhibiting protein synthesis. At a later

stage in germination, however, up to 72 hr, it is probable that RNA synthesis is essential. Barker and Hollinshead (1964) were unable to detect RNA synthesis in the pea seeds and actinomycin D had no effect; this does not necessarily mean that there is no RNA synthesis during the early germination of the pea seed—the results could have been due to the impermeability of the seedling at this stage. Barker and Rieber (1967), however, suggested that polysomes are synthesized during the first 17 hr germination of *Pisum arvense*. They failed to extract polysomes from the dry seed and electron microscopy of fixed, sectioned dry seeds suggested that polysomes do not exist at that stage. They found that the newly formed polysomes at 17 hr can be isolated only if zinc ion is used as a nuclease inhibitor in the grinding medium. After two or more days' germination, polysomes were obtained even without zinc ions. This is more probably due to some change in the nuclease sensitivity of the polysomes, than to a change in the nuclease content of the cells. The yields of polysomes they obtained were small in all cases. R. L. Watts and A. P. Matthias (unpublished results) found that barley seed, germinated for one day, also contained polysomes which were more sensitive to nucleases than those obtained after long periods of germination.

Marcus and Feeley (1964) found that the ability of the isolated ribosomes to incorporate amino acids into protein increased during germination; in this case, and that of Barker and Rieber (1967), the ribosomes from the dry seed could be stimulated to incorporate phenylalanine with added polyuridylic acid, suggesting that they were functional but lacked only messenger RNA. The absolute levels of incorporation obtained by the two groups were very different, however. This, and the possible over-riding effect of poly U over natural messengers, makes the interpretation of their experiments tentative.

There are clearly three simple possibilities for the mechanism of the onset of protein synthesis during germination: either polysomes are present at all times, even in the dry seed, and protein synthesis starts with the general increase of metabolism in the cell; or polysomes are formed using pre-existing messenger RNA in the cytoplasm; or there is a requirement for new messenger and/or other RNA synthesis. I would like to suggest that these alternatives are not mutually exclusive but that more than one mechanism can operate at one time. It is also possible that different mechanisms operate either in different species of plant or even in different batches of the same species depending on physiological differences during ripening and storage of the seed. Perhaps a clue to this view is provided by the experiments of Waters and Dure (1966) in which RNA synthesis could be detected in the germinating seed but could be inhibited without apparently affecting other processes. The extent to which messenger is stored in the cytoplasm, and the form in which it is stored, may thus vary. This would explain the somewhat conflicting preliminary results reported above.

V. Polysomes in Chloroplasts

The above discussion has been about polysomes in the cytoplasm of plant cells with the assumption that the RNA of these polysomes is synthesized in the nucleus. It is clear now that chloroplasts contain another class of ribosomes (Eisenstadt and Brawerman, 1964; Sissakian *et al.*, 1965) and a few recent papers have shown that these also can occur as polysomes. The stability of isolated chloroplast polysomes is probably even lower than those from the cytoplasm and there is likely to be considerable variation between species in this respect as judged by the stability of the RNA (Loening and Ingle, 1967). Clark (1964) found evidence for the existence of chloroplast ribosomes and showed that their presence was dependent on light. Chen and Wildman (1967) have shown that isolated chloroplast polysomes are active in amino acid incorporation *in vitro* into protein. The most exacting density gradient analysis of chloroplast ribosomes, in comparison to cytoplasmic polysomes, has been carried out by Stutz and Noll (1967). They showed clearly for the bean leaf that the chloroplast polysomes are polymers of the 70S ribosome and that the cytoplasmic polysomes are polymers of the 80S ribosomes. The superb gradients of mixtures of the two show unequal spacing and irregular heights of the peaks due to overlap of the narrower chloroplast polysome spacing with the wider cytoplasmic particles. The yields they obtained were small and the time of preparation of the extracts was long (about 4 hr) and no special nuclease inhibitors were used. It is remarkable that the polysomes survived for so long which suggests that the bean (Pinto) is a suitable species for further study.

These experiments and those on the isolated RNA (Loening and Ingle, 1967; Stutz and Noll, 1967) show that the protein synthesizing system of the chloroplasts is distinct from that of the cytoplasm. It should now be possible to investigate the separate control of the two systems and determine, for example, whether the chloroplast ribosomes can use nuclear messenger RNA.

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