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Polyribosomes from Peas

V. AN ATTEMPT TO CHARACTERIZE THE TOTAL FREE AND MEMBRANE-BOUND POLYSOMAL POPULATION

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ABSTRACT

Attempts were made to isolate and characterize the total population of free and membrane-bound polysomes from the elongating region of dark-grown pea stems (*Pisum sativum* L.). Partial separation of free from membrane-bound polysomes was achieved by relatively low speed centrifugation of the homogenate. Complete separation was not achieved. Based on analysis of the rRNA content of various subcellular fractions, fractionated tissue yielded greater than 95% of the rRNA found in whole tissue. Approximately 45% of the ribosomal material was membrane-bound (released by detergent) and was found in the "wall" (13%), the "nuclear" pellet (2%), and the "mitochondrial" pellet (29%). The remaining 55%, consisting primarily of free polysomes, could be recovered free from membranous material by sedimentation through a dense (700 mg/ml) sucrose pad for 90 hours. The advantages and disadvantages of using sucrose pads for the separation of free and membrane-bound polysomes are discussed.

In earlier papers we described techniques for isolating free polysomes from peas (16) and showed that the growth hormone, indoleacetic acid, caused a marked increase in this class of polysomes soon after it was applied to aged pea tissue (14). However, we recognized the potential importance of MBP[1] in this system, because the enzymes implicated in growth (wall-softening) must be secreted through the cytoplasmic membrane to attack their substrates. These enzymes might, therefore, be associated with rough endoplasmic reticulum, golgi bodies, or other organelles as has been found with secreted enzymes in animal tissues (27).

The most commonly used method employed to isolate MBP from animal tissues has been to layer a PMS on a discontinuous gradient and collect the membranous material held up by the heavier layer (1). More recently, however, a number of workers have shown (2, 4, 20, 25, 26, 32, 33) that the majority of MBP can be found in the mitochondrial pellet, rather than in the PMS. Relatively little work has been reported with MBP from plant tissue (12, 23, 28).

This paper describes methods used in an attempt to characterize the total population of both free and membrane-bound polysomes from a plant tissue as a prerequisite to finding out how the hormone affects their metabolism.

[1] Abbreviations: MBP: membrane-bound polyribosomes; PMS: postmitochondrial supernatant.

MATERIALS AND METHODS

Methods for isolation of free polysomes have been described previously (16). Apical 10-mm segments from the third internode of dark-grown Alaska pea seedlings (*Pisum sativum* L.) were ground in an ice-cold mortar in 5 volumes of buffer A (0.2 M tris-HCl, pH 8.5; 0.2 M sucrose; 60 mM KCl; 30 mM $MgCl_2$). The resulting brei was strained through nylon cloth to retain the "wall" fraction, and the liquid was centrifuged for 10 min at 29,000*g* to yield a "mitochondrial" pellet. The PMS was layered on a 4-ml sucrose pad of 700 mg/ml sucrose in buffer B (40 mM tris-HCl, pH 8.5; 20 mM KCl; 10 mM $MgCl_2$) and centrifuged for 2 or more hr at 95,000*g* in the 40 rotor of a Spinco Model L ultracentrifuge. Polysome profiles were prepared by either layering the polysome pellet resuspended in buffer B or the postmitochondrial supernatant on linear (150–600 mg/ml) sucrose gradients and centrifuging for 75 to 120 min at 122,000*g* (avg) in a SW-36 rotor. Gradients were scanned with an ISCO Model 640 gradient fractionator, and the A_{254} was monitored continuously. Areas under different peaks were measured by planimetry.

Membrane-bound polysomes were prepared by two methods (1, 2, 4, 7–9, 20, 25, 26, 32, 33). Homogenate which had been filtered through nylon cloth was centrifuged at 500*g* for 10 min and the supernatant was recentrifuged at 29,000*g* for 10 min. The resulting pellet was resuspended in buffer A and recentrifuged at 29,000*g* to remove contaminating free polysomes. This pellet was resuspended in buffer A or buffer B ± 1% Triton X-100, and an aliquot was centrifuged on polysome gradients. Alternatively, the post 500*g* supernatant was layered on a discontinuous sucrose gradient consisting of 2 ml of 700 mg/ml sucrose overlayed with 2 ml of 350 mg/ml sucrose in buffer B. After centrifuging for 1 hr at 95,000*g* in the 40 rotor, the membranous material at the lower interface was removed by aspiration, diluted, and centrifuged at 29,000*g* to produce a membranous pellet. Polysomes were then prepared by techniques described for the original 29,000*g* pellet.

RNA extractions were made by homogenizing whole tissue or subcellular fractions in GPS buffer (0.1 M glycine, 0.3 M NaCl; 50 mM K_2HPO_4, pH 9.4) (10, 17). The GPS-treated homogenate was centrifuged at 29,000*g* and the supernatant layered over linear (75–300 mg/ml) sucrose gradients in GPS buffer and centrifuged for 14 hr at 67,500*g* (avg) in a SW-36 rotor. Gradients were scanned as above and the area of the large and small rRNA species measured by planimetry. In addition, RNA was measured by A_{260} to A_{280} readings of perchloric acid extracts.

RESULTS

Localization of rRNA in Subcellular Fractions. The techniques used routinely in this (14–16, 19) and many other labo-

ratories (5, 11, 18, 21, 22, 30, 31, 35) for the isolation of polysomes from plants have involved grinding tissue in a buffer which resists RNase action, removing mitochondria and other organelles by low speed centrifugation, and pelleting the ribosomes from the PMS through a pad containing sucrose at 1.5 to 2 M. Alternatively, the PMS has been layered directly onto gradients (29, 34) or the ribosomes pelleted in the absence of a pad (12, 23, 24, 28). The MBP are thought to be held up by the dense pad (7–9) and thus effectively separated from the free polysomes.

We attempted to isolate MBP from the PMS by collecting them above a 2 M sucrose pad, but we found very few present (*e.g.* Fig. 7, C and D). Because of this and because recent studies with animal tissues had shown that the majority of MBP are pelleted with the mitochondria (1, 2, 4, 20, 26, 32, 33), we decided to analyze the RNA content of each centrifugal fraction to find out where significant amounts of RNA were located. Instead of relying solely on extraction of total RNA from the various fractions (20, 26, 33), we also used a method (10, 17) which permits measurement of ribosomal RNA (extraction in GPS buffer prior to separation of rRNA on gradients) as this would give a more direct quantification of ribosome content.

The profiles depicted in Figure 1 show the rRNA extracted from fractions designated as the "4000*g* pellet" (Fig. 1A), the "29,000*g* pellet" (Fig. 1B), "95,000*g* pellet" (Fig. 1C), and whole tissue (Fig. 1D). Ribosomal RNA was present in all fractions and since the ratio of large to small rRNA was always 2.05 to 2.15 it appeared as though equimolar amounts of these two species had been obtained in undegraded form (13). The data from Figure 1 and from other similar experiments are summarized in Table I and show the yield of rRNA from each fraction in A_{254} units/g tissue. An appreciable amount (13%) was found in the wall fraction, a minor amount (2%) in the 500*g* pellet, the greatest amount (29%) in the mitochondrial fraction, and a substantial amount (14%) in the polysome pellet. However, the sum of the individual fractions is less than 60% of the amount recovered from the whole tissue. Similar results were obtained with perchloric acid extraction (data not shown). The most logical location for the missing 40% was in the fractions not pelleted from the PMS and so this fraction was studied first.

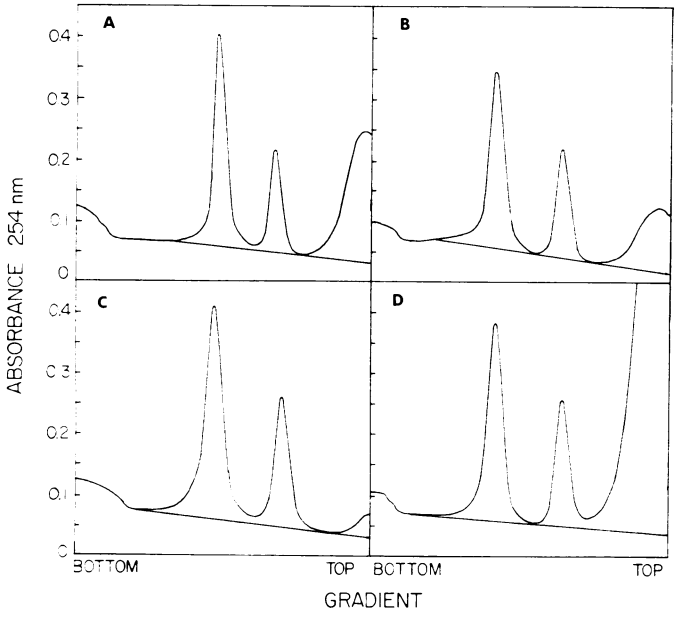

FIG. 1. Absorption profiles of rRNA extracts from fractionated and whole tissue. Apical 10-mm segments from the third internode were ground in buffer A. The homogenate was strained through nylon cloth to retain the "wall" and then centrifuged at 500*g* for 10 min to obtain a "nuclear" pellet. The supernatant was recentrifuged at 4000*g* for 10 min, transferred to a second tube, and centrifuged for 10 min at 29,000*g* to obtain a mitochondrial pellet. The PMS was layered over a 700 mg/ml sucrose pad in buffer B and centrifuged for 3 hr at 95,000*g* in the rotor of a Spinco Model L ultracentrifuge to pellet polysomes. RNA extractions were made by homogenizing pellets or whole tissue in GPS buffer and centrifuging the extract for 10 min at 29,000*g*. The supernatant was layered on linear (75–300 mg/ml) sucrose gradients in GPS buffer and centrifuged at 67,500*g* for 14 hr in a SW-36 rotor. Amount of tissue used for RNA extraction: A, 4000*g* pellet, 0.5 g; B, 29,000*g* pellet, 0.06 g; C, polysome pellet, 0.1 g; and D, whole tissue, 0.02 g.

Table I. *Subcellular Localization of Ribosomal RNA*

Fraction[1]	RNA[2]		Average (% of whole tissue)
	Exp. 1	Exp. 2	
	units/g tissue		
Whole tissue	49.00	48.60	100
"Wall"	6.81	5.84	13
500*g* pellet	0.93	0.97	2
29,000*g* pellet	13.70	14.64	29
95,000*g* pellet	6.30	6.70	13
Total	27.74	28.15	57
Not recovered	21.26	20.45	43

[1] Tissue fractionation was the same as in Figure 1, except the 4000*g* centrifugation was omitted.

[2] rRNA was isolated and separated on gradients as described in Figure 1, and the area of absorbance is expressed as planimeter units per g of tissue.

Analysis of Polysomes in PMS (Primarily Free Polysomes). Three different methods were used to check whether the missing material was, in fact, in the fractions of the PMS not pelleted by ultracentrifugation. First, the PMS was centrifuged for varying periods of time (up to 90 hr) over pads with constant sucrose concentration (700 mg/ml). Second, the PMS was centrifuged for a standard period of time (4 hr) over pads of varying sucrose cencentration (200–600 mg/ml). In both instances, the pellets were analyzed for both rRNA and polysome content. Third, the PMS was layered directly on gradients and the polysomes alone were analyzed.

Two major experiments were conducted using varying periods of centrifugation, the first using from 2- to 23-hr centrifugations (Fig. 2) and the other (when it was found that material was still pelleting at 23 hr) using 24- to 90-hr centrifugations (Fig. 3). There was a continued increase in material of all size-classes (monosomes to large polysomes) over the 2- to 23-hr time period (Fig. 2), but an increase primarily in monosomes and small polysomes over the 24- to 90-hr period (Fig. 3). The increase in yield of various polysome size-classes with increasing centrifugation time is shown in Figures 4 and 5. The data in Figure 4 show the increase in the amount of monosomes, di-, tri-, and tetramers as a ratio of their respective 2-hr value. The data in Figure 5 show the increase in actual ribosomal material (in A_{254} units/g tissue) for the monosomes (M), for polysomes greater than 5-mers (large polysomes, LP), and for the total ribosomal material (monosomes plus polysomes, M + P).

From Figure 4, it is apparent that the smaller particles pellet more slowly and are discriminated against when short term centrifugations are performed. This is especially true for the monosomes (and subunits which were not analyzed in this

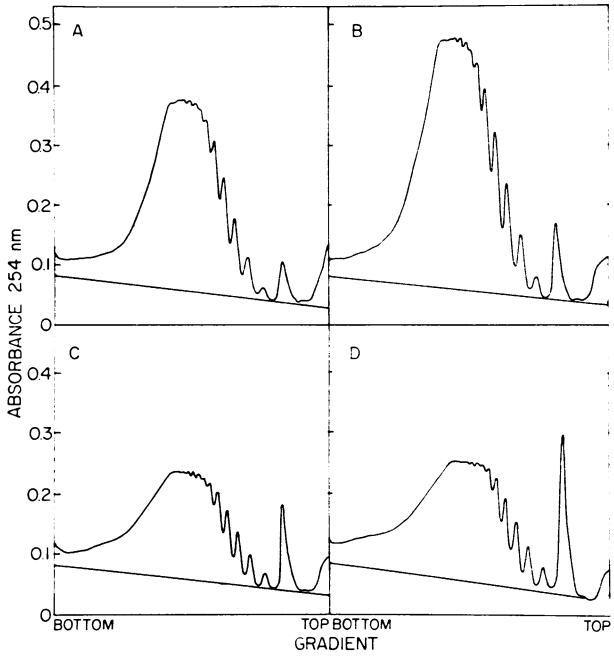

FIG. 2. Polyribosome profiles obtained after centrifuging the PMS for 2 to 23 hr. Methods of polysome isolation were the same as in Fig. 1. Fresh weight equivalent and length of centrifugation: A, 0.5 g, 2 hr; B, 0.5 g, 4 hr; C, 0.2 g, 10 hr; D, 0.2 g, 23 hr.

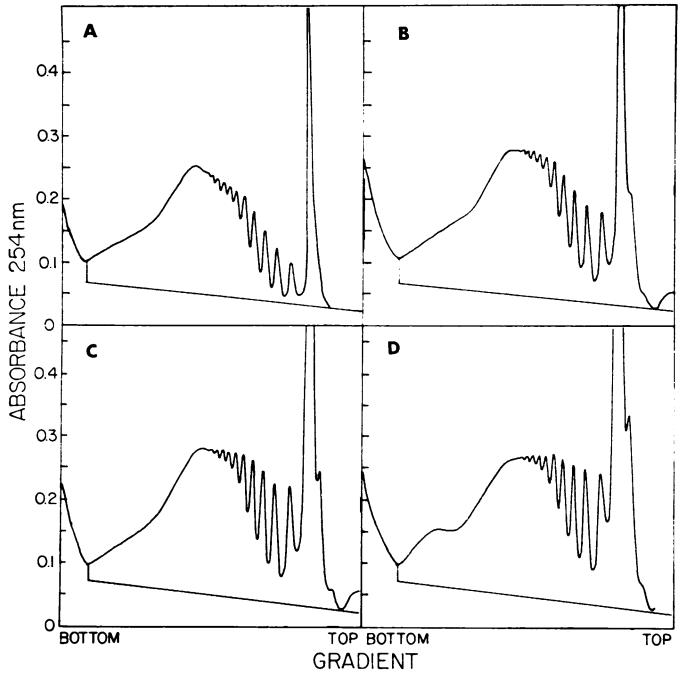

FIG. 3. Polysome profiles from PMS centrifuged 24 to 90 hr. Methods as in Fig. 2 except that extract from 0.15 g was layered in each case. Duration of centrifugation: A, 24 hr; B, 48 hr; C, 72 hr; D, 90 hr.

study). However, the data in Figure 5 show that the monosomes make up only a small proportion (10%) of the total material after 23-hr centrifugation and that, in terms of the amount of actual material, more large polysomes are pelleted with increasing centrifugation time up to 23 hr. A major change which occurred with the longer centrifugations was the change in polysome distribution from a normal population (Fig. 2A)

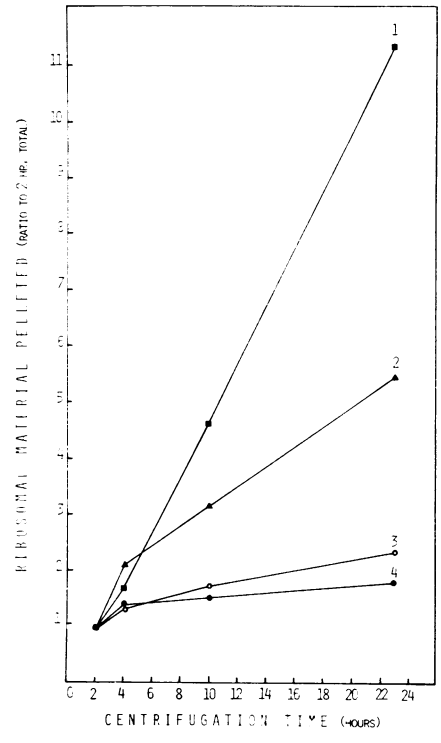

FIG. 4. Effect of centrifugation duration on yield of monosomes and small polysomes. The area of the polysome size-classes was determined by planimetry of profiles in Fig. 2. Size classes: 1, monosome; 2, dimer; 3, trimer; 4, tetramer.

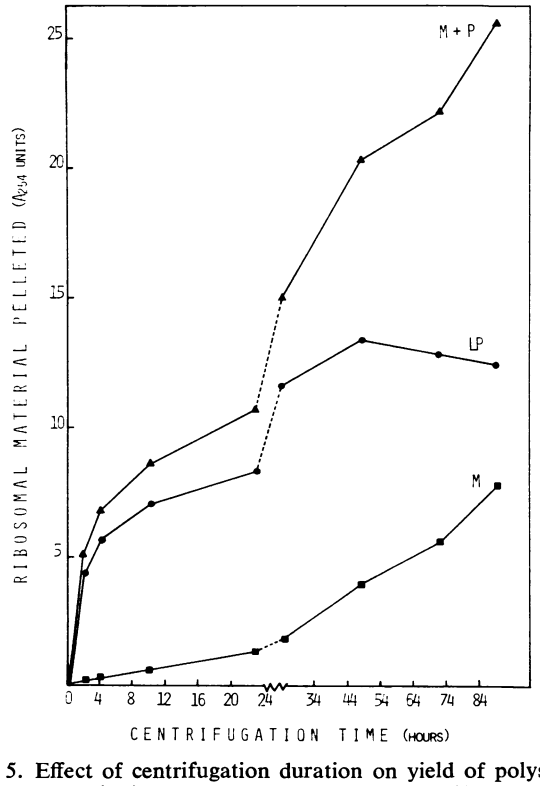

FIG. 5. Effect of centrifugation duration on yield of polysomes. The data were obtained from profiles (from two different experiments) depicted in Fig. 2 (2–23 hr) and Fig. 3 (24–90 hr). Size classes: M, monosome; LP, large polysomes (larger than 5-mers); M + P, total (monosomes plus polysomes).

to one with the dimer, trimer, and tetramer almost equal in area (Fig. 3D). Further work was carried out to determine whether the many small polysomes that appeared after 90-hr centrifugation were a reflection of the *in vivo* condition, or whether they were an artifact (of polysome degradation) occurring during the excessively long centrifugations. The possibility of degradation was suggested by the fact that there was actually a decrease in the amount of large polyribosomes with centrifugations longer than 48 hr (Fig. 5). The other main observation arising from these data is that there was four times as much ribosomal material pelleted after 90 hr (25 units) as there was after 2 hr (6 units). This difference (19 units) accounts for most if not all of the "missing" material (Table I). Furthermore, GPS extracts of identical polysomes pellets showed similar increases in rRNA with longer centrifugations (data not shown).

The second method involved centrifuging identical samples of PMS for 4 hr over pads of sucrose at 200, 400, and 600 mg/ml. The profiles shown in Figure 6 demonstrate that a decreasing amount of ribosomal material pelleted through pads of increasing concentration. There was a dramatic decrease

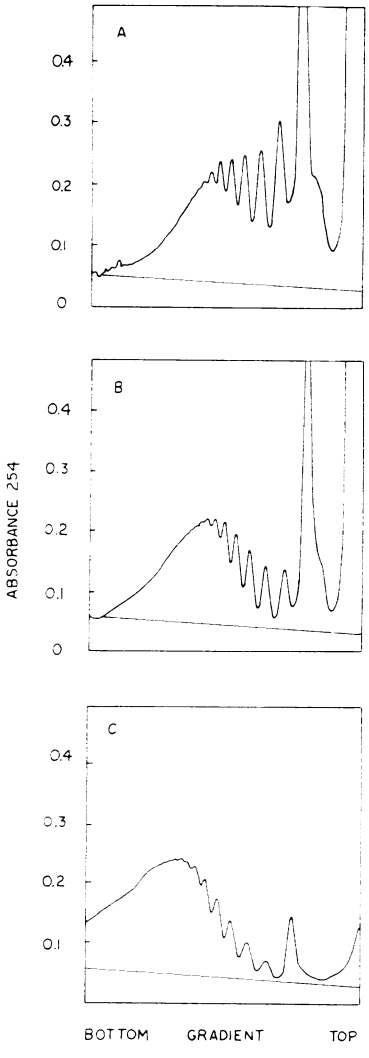

FIG. 6. Polysome profiles obtained after centrifuging PMS for 4 hr over sucrose pads of varying concentration. Fresh weight equivalent and concentration of sucrose in the pad: A, 0.18 g, 200 mg/ml; B, 0.18 g, 400 mg/ml; C, 0.28 g, 600 mg/ml. Polysome pellets from A and B were resuspended in buffer B containing Triton X-100 to solubilize the pellet.

in monosomes and small polysomes when heavier (600 mg/ml) pads were used (Fig. 6C) in place of lighter pads (200 and 400 mg/ml) (Fig. 6, A and B). However, these differences cannot be entirely the result of discrimination against smaller particles by the denser solutions because the amount of large polysomes increased when heavier pads were used. These large polysomes were RNase-sensitive and so were not an artifact of ribosomal aggregation (data not shown); therefore, some of the monosomes and small polysomes that pelleted through the lighter pads must be a result of degradation of the larger polysomes which remained intact when heavy pads were used. This provides further evidence (enhancing that obtained with longer centrifugations) that polysome degradation can occur during isolation. It seemed likely that this degradation was caused by factors (RNase) present in the membranous fraction, because (*a*) the heavier (600 mg/ml) pad prevented pelleting of membranes in 4-hr centrifugations and (*b*) the interface between the PMS and the pad gradually disappeared (by diffusion of sucrose) so that by 90 hr the membranous material was in close contact with the pelleted ribosomes.

We tested for the presence of RNase in the membranous fraction by obtaining samples of polysomes and samples of membranous material from above the interface and incubating them separately or together in the presence and absence of detergent (1% Triton X-100). The results of this experiment are shown in Figure 7. The free polysomes (Fig. 7A) were relatively unaffected by detergent treatment (Fig. 7B). The membranous material yielded a few small polysomes and monosomes in the absence of detergent (Fig. 7C), but in the presence of detergent it yielded many more monosomes and small polysomes (Fig. 7D). However, the mixture (free polysomes plus membranous material) was changed even more markedly by detergent treatment. In the absence of Triton X-100 (Fig. 7E), the profile appeared like the sum of the two component profiles (Fig. 7A plus 7C), whereas in the presence of detergent (Fig. 7F) the mixture yielded typically degraded polysomes. The conversion of large polysomes to smaller ones in the presence of the membrane fraction was analyzed by a mRNase assay (15) and shown to be identical to that caused by exogenous RNase (calculations not shown). Similar results were obtained when deoxycholate was substituted for Triton X-100. These studies confirm the existence of a membrane-associated RNase in pea tissue (6).

The third method used to examine the polysomes in the PMS was to separate them by direct application of the PMS to sucrose gradients using samples similar to those used for the prolonged centrifugation experiment shown in Figure 2. The profiles depicted in Figure 8 show that poor separation was obtained when normal periods of gradient centrifugation (75 min) were used (Fig. 8A), but resolution was enhanced when 120-min (Fig. 8B) centrifugations were employed. Furthermore, these data show that the PMS yielded a normal distribution of polysomes and the high amounts of monosomes and small polysomes obtained by prolonged centrifugation (Fig. 3, A–D) or light pads (Fig. 6, A and B) must have been artifactual. Unfortunately, it is difficult to measure the area of absorbance of the smaller particles (subunits, monosomes, and even dimers) because this region of the gradient is contaminated with UV-absorbing material from the supernatant. We have found that the most satisfactory way of analyzing the PMS directly is to use two samples, each with a postpolysomal supernatant as a blank. One of these samples should be centrifuged for about 2 hr to separate the polysomes, the other centrifuged for at least 4 hr to separate the smaller particles from the supernatant. Even when these precautions are taken, the exact positioning of the baseline is uncertain and the area of the monosomes and subunits cannot be analyzed with com-

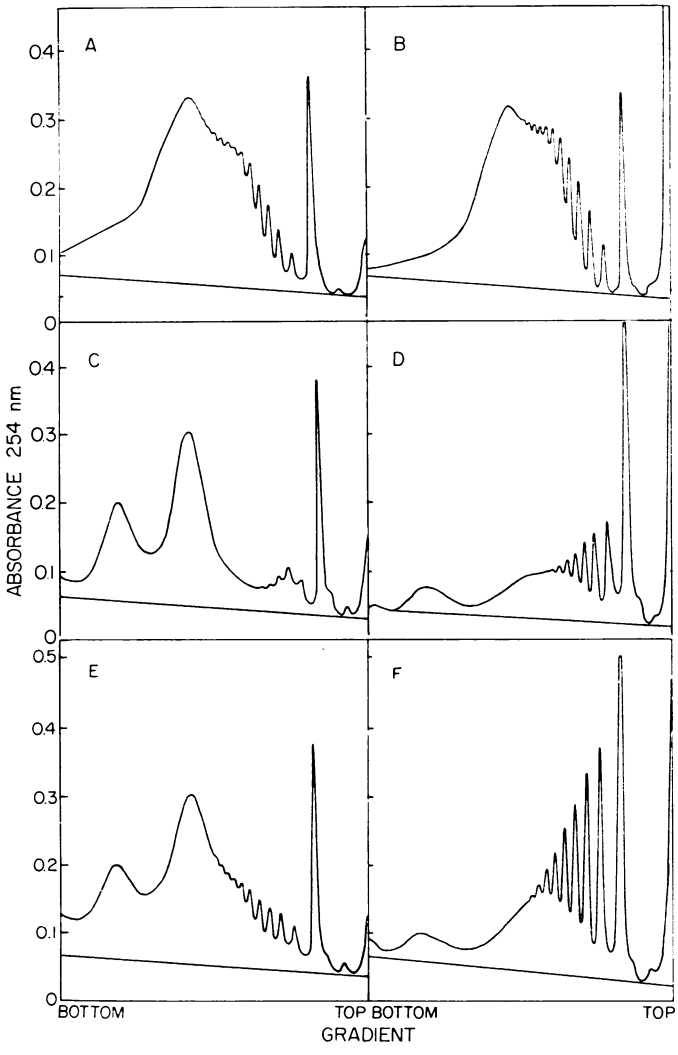

FIG. 7. Polysome degradation in the presence of detergent-treated membranes. Methods for polysome isolation were the same as in Fig. 1. Membranous material was obtained by centrifuging the PMS over a 700 mg/ml sucrose pad for 4 hr at 95,000*g* and collecting the material from above the interface. This was centrifuged at 29,000*g* for 10 min and the pellet retained. Pelleted polysomes and pelleted membranes were resuspended separately or together in the presence or absence of detergent and incubated for 2 hr at 2 C prior to layering on gradients. Amount of tissue used: A, B, polysomes, 0.25 g; C, D, membranes, 0.2 g; E, F, polysomes, 0.1 g, membranes 0.1 g. Triton X-100 (1%) was present in B, D, and F.

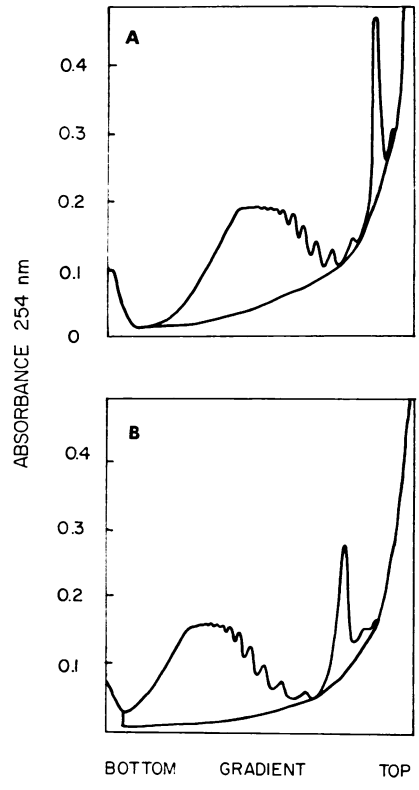

FIG. 8. Polysome profiles from PMS layered directly on gradients and centrifuged for different periods. Baselines were estimated by co-centrifugation of an equal amount of ribosome-free extract on identical gradients. Each gradient has the equivalent of 40 mg fresh weight. Duration of centrifugation: A, 75 min; B, 120 min.

plete confidence. A polysome yield of 25 to 26 units/g tissue was obtained using these methods (Fig. 8, A and B), which was virtually identical to that obtained after 90-hr centrifugation of the PMS (Fig. 5). This supports the suspicion that most, if not all, of the "missing" material was free polysomes which did not sediment quickly through heavy sucrose pads, and the total free polysomes (25–26 units) comprise about 55% of the total polysome population.

The major conclusion that can be reached based on the results of the three main types of experiments employed (prolonged centrifugation through heavy pads, sedimentation through dilute pads, direct layering of PMS onto gradients) is that there is no easy way of separating the entire population of free ribosomes in an undegraded condition from the membranous material in the PMS. A short term centrifugation (up to 10 hr) through a 600 to 700 mg/ml pad permits the isolation of undegraded, free polysomes, but only a part (30%) of the total number is recovered and the larger polysomes are obtained preferentially. Longer term centrifugation (>24 hr) permits the isolation of a greater proportion of the total population, but these polysomes are increasingly degraded and may contain membrane-bound polysomes (because the interface is lost through diffusion). In spite of the difficulties encountered with the use of heavy pads, such pads must be used to prevent degradation caused by the nucleases in the membranes which co-pellet with the polysomes through lighter pads and to prevent contamination of the free polysomes with MBP. If, however, contamination of the free polysome fraction with the small amount of membrane-bound polysomes present (as in the PMS from peas) is not a major concern, direct layering of PMS onto sucrose gradients would be the recommended procedure, especially when the total free polysome population is under investigation.

Analysis of Polysomes in Mitochondrial Fraction (Primarily Membrane-bound Polysomes). When few MBP (held up by a sucrose pad) were found in the PMS and when about 30% of the total ribosome population was found in the mitochondrial pellet (Table I), we attempted to analyze this pellet to find out if it contained MBP. Our initial attempts to characterize the polysomes in this fraction were unsuccessful. When the membranous material was resuspended in buffer B (low ionic strength) without detergent, most of the material appeared as a major peak sedimenting four-fifths of the way down the gradient, with only a few small polysomes and monosomes apparent (Fig. 9A). When 1% Triton X-100 was added, the major (membranous) peak disappeared and many polysomes appeared, but they were partially degraded (Fig. 9B). Once more, it seemed as though RNase was present in the mem-

branous material. Our earlier attempts at extraction of polysomes from whole tissue (16) had shown that a buffer of high ionic strength (buffer A) was necessary to diminish RNase action, and so we used buffer A to isolate polysomes from the 29,000*g* pellet. With this buffer in the presence of detergent, there was little or no degradation and a normal polysome distribution was obtained (Fig. 9C). That this material was indeed polysomal is supported by the fact that a combination of detergent plus high levels of exogenous RNase completely removed the faster sedimenting material (Fig. 9D).

Like many others (1, 25, 26, 33), we were unable to determine exactly what proportion of the polysomes that sediment at 29,000*g* were genuinely membrane-bound. This fraction has a large number of ribosomes associated with it (Fig. 1B, Table I) which can be seen under the electron microscope. Some of the ribosomal material seems to be trapped within membranes, some seems to be either genuinely membrane-bound or else consists of free polysomes adsorbed onto membranes (data not shown). We attempted to purify those polysomes most firmly associated with the membranes by repeated resuspension and repelleting of the material. The profiles in Figure 10 show that the amount of polysome material decreases with each successive cycle of pelleting, but the qualitative changes are less apparent. Routinely, we now use material that has been resuspended in buffer A and repelleted once prior to treatment with detergent and layering onto gradients. Similar yields of primarily membrane-bound polysomes can be obtained by layering the post-500*g* supernatant directly over a 700 mg/ml sucrose pad and collecting the material held up by the pad. Unfortunately, the sedimentation of free polysomes is slowed by these heavy pads and we were never satisfied that we had achieved clear separation of the free polysomes from the MBP.

The other major fraction containing significant amounts of polysomal material is the "wall" fraction—the material retained after pressing the homogenate through nylon cloth. This material is very similar to the material sedimenting at 29,000*g*, *i.e.*, it has a normal distribution of polysomes when extracted in the presence of high ionic strength buffers, and is thus apparently membrane-bound. We have not been able to determine whether the polysomes are derived from unbroken cells, from membranes (plasmalemma?) associated with the wall, or from some other membranous component. This fraction is, however, of some importance to us as it contains high levels of cellulase activity after treatment of tissue with IAA (as does the 29,000*g* pellet) and this enzyme is most likely involved in wall metabolism.

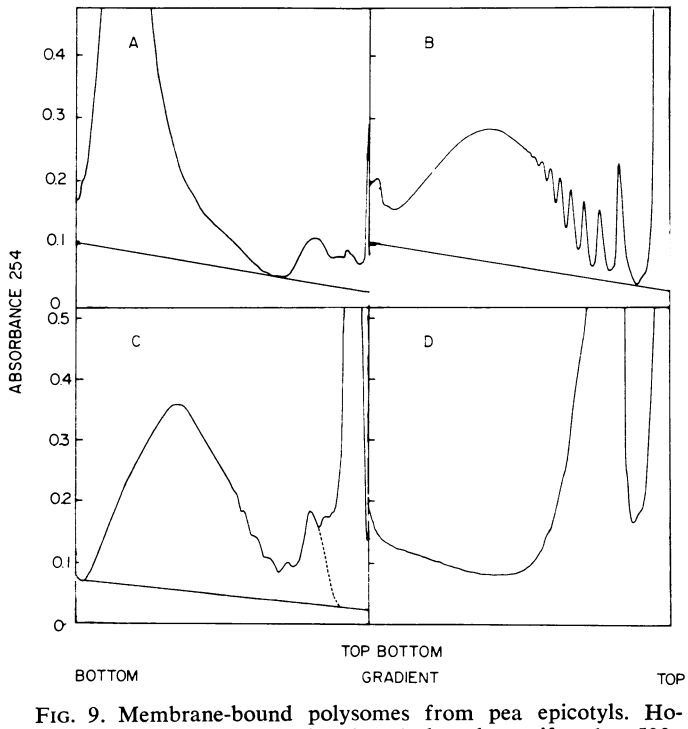

FIG. 9. Membrane-bound polysomes from pea epicotyls. Homogenate was strained through nylon cloth and centrifuged at 500*g* for 10 min and the supernatant recentrifuged at 29,000*g* for 10 min. The pellet was resuspended in buffer and the equivalent of 0.5 g fresh weight layered directly onto gradients. Resuspension buffers: A, buffer B; B, buffer B plus 1% Triton X-100; C, buffer A plus 1% Triton X-100; D, buffer A plus Triton plus 100 μg RNase.

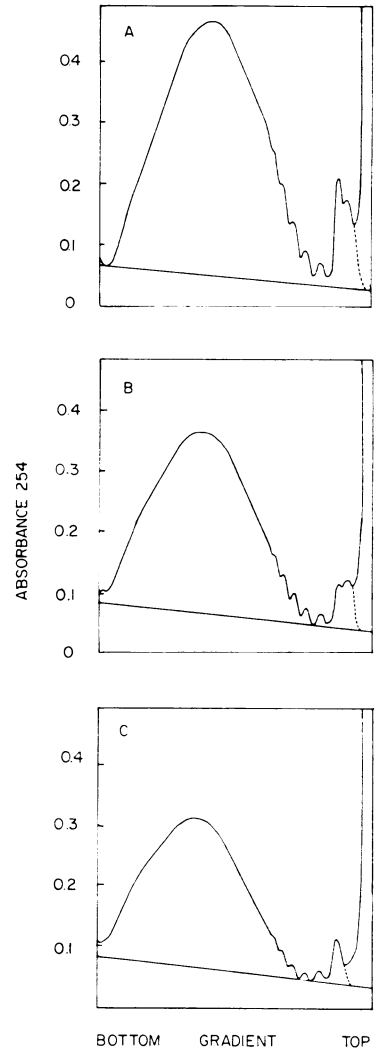

FIG. 10. Purification of MBP from pea epicotyls. MBP were prepared by methods described in Fig. 9, except that the initial 29,000*g* pellet was resuspended in buffer A and repelleted at 29,000*g* for the additional times; A, none; B, once; C, twice.

DISCUSSION

The use of a sucrose pad in the purification of polysomes seems to have originated with Noll and co-workers. In their original report (36) they showed that heavier pads decreased the amount of polysome material that pelleted, but yielded a fraction more active in protein synthesis *in vitro*. Bloemendal *et al.* (8) were apparently the first to use a sucrose pad for the retention of MBP and their methods have been used frequently

in studies with animal tissues. Many of these studies have been criticized recently (1, 2, 4, 20, 25, 26, 32, 33) on the grounds that the majority of the MBP were discarded with the mitochondrial pellet. In the rather scant literature on MBP in plants, Bloemendal's methods have been used but the fraction sedimenting at 13,000*g* for 15 min (28), 19,000*g* for 30 min (12), or 38,000*g* for 15 min (23) was discarded. From the data presented here (Table I, Fig. 9) and from a number of recent reports on animal tissues (1, 20, 33), it would appear that the majority of the MBP had been discarded in these earlier plant studies.

Many of the studies, including ours (14), on free polysomes from plant tissues (5, 11, 18, 19, 21, 22, 30, 31, 35) have followed Bloemendal's method of removing the MBP with a sucrose pad, without realizing that its original use was to isolate large polysomes selectively (3, 36). From the data presented here (Figs. 2–5), it is apparent that most of the studies on free polysomes from plants can be criticized on the grounds that the high speed pelleting of polysomes through a pad was not long enough and as much as 70% of the free polysomes might not have pelleted (Fig. 5). Occasions do exist, however, when the discriminatory effect of the sucrose pad is advantageous: first, in its original use, to obtain more active *in vitro* protein synthesis (3, 36); second, when polysomes are used as a substrate for RNase (15); and third, whenever free polysomes must be completely separated from membranous material.

It is only recently that a number of "balance sheet" studies have been carried out with animal tissues to determine the subcellular localization of RNA as a preliminary attempt to localize polyribosomes (12, 26, 33). To our knowledge, there have been no comparable studies on plant tissues prior to this. In general, our findings are similar to those reported with animal tissues, *i.e.*, the majority of MBP sediment at quite low speed and partial separation of free and MBP can be achieved quite easily, although complete purification of the two classes of polysomes is far more difficult. One facet about our study that is different from previous ones is that we used GPS extraction and gradient separation to obtain ribosomal RNA yields so that we could estimate the ribosome content more directly.

The methods we would recommend for fractionation of free and MBP from plant tissues are essentially the same as those used with mouse myeloma cells (4). The tissue should be homogenized in a buffer which prevents RNase action, the debris removed by straining through cloth or by very low speed centrifugation. The MBP should be pelleted by centrifugation for about 10 min at about 30,000*g*, the pellet washed once and then resuspended in detergent in a buffer resistant to RNase, prior to separation on gradients.

The free polysomes in the PMS should be separated by direct layering onto gradients and centrifuged for a sufficient period of time to permit adequate resolution. However, if membranous material must be removed, we would recommend use of a pad of at least 1.5 M sucrose and a period of centrifugation longer than 10 hr and shorter than 24 hr as a compromise between maximal yield and minimal degradation.

We are currently using these techniques (analysis of MBP in the 29,000*g* pellet and analysis of the total free polysomes by direct layering of PMS onto gradients) to re-assess the conclusions we made earlier (14), *i.e.*, that a 2-fold increase in polysomal mRNA occurred within 6 hr of application of a hormone to aged pea tissue. These conclusions (14) were based on samples which contained only 25% (Fig. 5) of the free polysome population and less than 15% (Table I) of the total rRNA content. The conclusions could have been valid, or they could be an artifact of the techniques employed, because, for instance, the hormone may have caused attachment of monosomes and small polysomes to membranes at the same time that it caused release of large polysomes from membranes.

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