Phytochemistry, 1968, Vol. 7, pp. 673 to 681. Pergamon Press. Printed in England

THE EFFECT OF 3-INDOLYLACETIC ACID ON THE LEVELS OF POLYSOMES IN ETIOLATED PEA TISSUE

ANTHONY TREWAVAS

School of Biological Sciences, University of East Anglia, Norwich, NOR 77H, Norfolk

(*Received* 22 *November* 1967)

Abstract—It is shown that after 12 hr incubation, the growth hormone 3-indolylacetic acid has increased the level of RNA in the post-mitochondrial supernatant of excised etiolated pea-stem tissue. Sucrose density gradient centrifugation revealed that part of this increase was in the ribosome population and in particular in the levels of polysomes. Sucrose also appears to increase the level of polysomes in this tissue. In this excised tissue the level of polysomes rapidly declines whilst the level of the monomeric ribosomes remains constant when the tissue is incubated on water alone. Evidence is presented to show that auxin increases the level of polysomes by enhancing their rate of synthesis and this arises from an alteration in the rate of synthesis of ribosomal RNA rather than the "messenger".

IN A previous paper[1] we were able to demonstrate by the technique of DNA/RNA hybridization that the prime change in RNA metabolism after treatment of pea tissue with IAA was in the synthesis of ribosomal RNA. The synthesis of "messenger" RNA was increased to a slight degree and there appeared to be a slight specific alteration in the synthesis of this fraction. Since the only criterion of "messenger" RNA in that paper was based on its hybridization properties it was decided that this rather surprising result should be checked using an alternative technique. Methylated albumin chromatography[2] (MAK) was chosen since a fraction which is probably at least in part messenger RNA has been well characterized in plants using this technique.[3–5] Batches of fifty pea-stem sections were incubated in ^{3}H or ^{14}C-adenosine for 2 hr, 3-indolylacetic acid (IAA) being added to the batch containing ^{14}C-adenosine. At the end of the incubation period both batches of tissue were bulked and the nucleic acid prepared and separated on MAK columns. The ratio of $^{14}C/^{3}H$ disintegrations could then be determined in each fraction so that if auxin had enhanced the synthesis of any particular fraction this would be detected by a higher $^{14}C/^{3}H$ than the remaining fractions. Such a system is thus internally controlled. The results are shown in Fig. 1. Only fractions 60–140 are shown in the figure since it is in these fractions that ribosomal and messenger RNA are eluted. The DNA and the two ribosomal RNA peaks can be clearly located from the $O.D._{260}$ profile and the messenger as the highly-labelled back peak. The $^{14}C/^{3}H$ ratio is lowest (*ca.* 0·5) over fractions 110–130 and this material is wholly messenger RNA. The ratio is at its highest (*ca* 1·05) over the second of the two ribosomal RNA peaks and at an intermediate value (*ca.* 0·65) over the first ribosomal peak. The counts for DNA labelling have not been included in this graph since they were too low for accurate determination. This experiment was repeated using slightly different conditions. Tissue was

[1] A. J. TREWAVAS, *Arch. Biochem. Biophys.*, in press.
[2] J. D. MANDELL and A. D. HERSHEY, *Anal. Biochem.* **1**, 66 (1960).
[3] J. INGLE, J. L. KEY and R. E. HOLM, *J. Mol. Biol.* **11**, 730 (1965).
[4] C. Y. LIN, J. L. KEY and C. E. BRACKER, *Plant Physiol.* **41**, 376 (1966).
[5] R. B. VAN HUYSTEE and J. H. CHERRY, *Biochem. Biophys. Res. Commun.* **23**, 835 (1966).

preincubated for 2 hr in the presence and absence of IAA and the labelled adenosine then being added for the third hour. This resulted in a marked enhancement of the response. The $^{14}C/^{3}H$ ratio of the first ribosomal peak was 1·0, the second ribosomal peak 1·7 and the so-called messenger 0·7. The results do again imply that auxin enhances particularly the synthesis of ribosomal RNA with a marked effect on the synthesis of the heavier ribosomal RNA fraction.

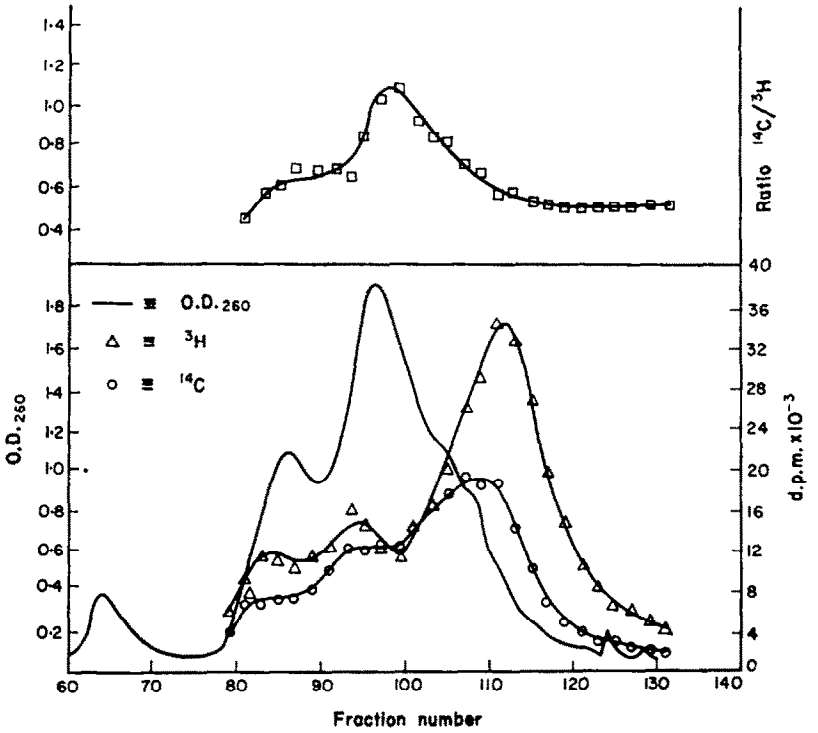

FIG. 1. METHYLATED ALBUMIN CHROMATOGRAPHY OF LABELLED RNA FROM IAA-TREATED AND UNTREATED TISSUE.

Batches of fifty epicotyl sections were incubated in 200 μc [^{3}H]-adenosine and 20 μc [^{14}C]-adenosine $+5\times10^{-5}$ M IAA for 2 hr. After incubation the two batches of epicotyls were bulked and 6 g sub-apical pea-stem sections added as carrier. The nucleic acid was prepared and separated on the column. The O.D.$_{260}$ and the disintegrations due to ^{3}H and ^{14}C were determined in each fraction. For convenience only fractions 60–130 are shown in the graph since it is this region that the relevant peaks are eluted. Disintegrations over the DNA peak are not shown since they were too low for accurate determination.

The results of Fig. 1 are in agreement with the data of Ingle and Key.[6] They were able to show that 2,4-dichlorophenoxyacetic acid increased the synthesis of ribosomal RNA to a much greater degree than "messenger" in soybean hypocotyl.

Previously[1] we were able to demonstrate that, in the first few hours after its addition, auxin failed to change the net level of RNA in this tissue. Since ribosomal RNA accounts for about 75 per cent of the total cellular RNA this implied that auxin may increase the net level of cellular RNA after a longer time-period than that initially measured. Evidence on this point is shown in Table 1. Batches of epicotyl sections were incubated for 12 hr in the

[6] J. INGLE and J. L. KEY, *Plant Physiol.* **42**, 29 (1965).

presence and absence of auxin and sucrose. The levels of RNA and protein were then assayed in the post-mitochondrial supernatant. The results show that there are small changes in the level of RNA in the post-mitochondrial supernatant but not in protein. The size of the auxin response (27 per cent in the absence of sucrose and 17 per cent in the presence of sucrose) in the RNA levels are similar to those noted by Key [7] in the excised soybean hypocotyl growing in 2,4-dichlorophenoxyacetic acid. The majority of RNA in the post-mitochondrial supernatant is present as ribosomal RNA. The results shown in Table 1 thus suggest that auxin may have increased the level of the ribosomes in this tissue. Accordingly, the effect of auxin on the level of the polysomes and monosomes has been studied.

Polysomes were isolated and separated on density gradients as described in the methods section. The addition of ammonium ion (final concentration 0·25 M kindly suggested by Professor Korner) to the homogenization medium was found to considerably enhance the stability of the polysomes. The proportion of ribosomes found in polysomes was increased from 55 per cent in the absence of NH_4Cl to 75 per cent in its presence. In addition, a pro-

TABLE 1. THE EFFECT OF IAA ON THE LEVEL OF RNA AND PROTEIN IN THE POST-MITOCHONDRIAL SUPERNATANT

Incubation conditions	RNA (mg)	Increase due to IAA (%)	Protein (mg)	Increase due to IAA (%)
1% sucrose + 8×10^{-5} M IAA	0·14	17	1·98	0
1% sucrose	0·12		1·98	
8×10^{-5} M IAA	0·14	27	2·08	4
Control	0·11		2·00	

1 g fresh weight of etiolated pea-stem sections were incubated for 12 hr at 25° in the above media. The tissue was then chilled and ground in 4 ml 0·4 M sucrose and spun at 20,000 *g* for 10 min. The supernatant was removed and an equal volume of 5% TCA added. RNA and protein were estimated on the precipitate and expressed as mg/g initial fresh weight.

nounced sharpening of the peaks was observed and the $O.D._{260/280}$ of the polysomes was increased from 1·85 to 1·99 and for the monosomes 1·86 to 1·92. Figure 2a shows a typical separation of polysomes isolated from fresh epicotyl tissue. The largest peak represents the monomer and this was shown by isolating monomeric ribosomes by the method of T'so, Bonner and Vinograd [8] and separating on density gradients. If the *s* value of the monomer is taken to be 74,[8] then the *s* values of the next three peaks (presumably dimer, trimer and tetramer) may be calculated to be 114–117, 145–147 and 168–171 in good agreement with the data obtained by other workers on plant polysomes.[4] The gradients shown in Fig. 2 have been deliberately overrun in order to effect adequate separation of the monomer from the dimer and a peak of material running behind the monomer. This results in the packing of the very heavy polysomes at the bottom of the gradients. The material running behind the monomer has an *s* value of 54–56 and probably represents the larger of the two sub-units of the ribosome which, according to T'so, Bonner and Vinograd,[8] has an *s* value of 58. The sub-unit does not appear to derive from the degradation of the ribosomes during the gradient separation since the simple expedient of layering the post-mitochondrial supernatant over

[7] J. L. KEY, *Plant Physiol.* **39**, 365 (1964).
[8] P. O. P. T'SO, J. BONNER and J. VINOGRAD, *J. Biophys. Biochem. Cytol.* **2**, 451 (1956).

6–7 ml 1 M sucrose instead of 4–5 ml (see Methods Section) results in almost total loss of this material. The absence of the smaller sub-unit in this case is probably because it fails to be centrifuged down under the conditions used. It was not found possible to reproduce separations exactly on different gradients, but the proportion of material running in the polysome region between different batches of peas was found to be extremely constant. For example, assay of the polysomes of four batches of freshly cut pea-stem sections taken from the same tray showed that the mean ± standard deviation of the percentage of ribosomes as polysomes was 74 ± 1 per cent.

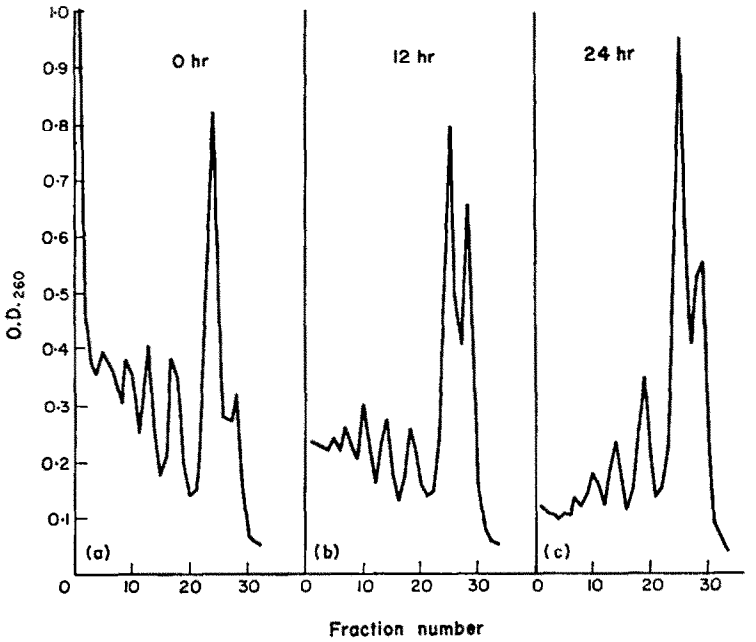

FIG. 2. THE EFFECT OF INCUBATING PEA-STEM SECTIONS ON WATER ON THE LEVELS OF POLYSOMES. Batches of fifty epicotyl sections were incubated on water at 25° for 0, 12 and 24 hr in darkness. The polysomes were then isolated and separated as described. Figure 2a shows the zero time sample, 2b the 12 hr incubation sample and 2c the 24 hr. The values for the total $O.D._{260}$ material on the gradient were in the zero value, 10·61, 12 hr value 8·61, 24 hr value 7·81. This represents a total loss of nucleic acid of 15 per cent in the first 12 hr and 26 per cent in the first 24 hr.

Figure 2a–c shows the effect on the ribosome population of incubating excised stem sections of etiolated pea tissue for 0, 12 and 24 hr and the data of the three gradients has been summarized in Fig. 3. It can be seen that as the tissue ages the level of material running in the polysome region shows a rapid decline, particularly in the heavy polysomes. The monosome, on the other hand, remains relatively constant but the level of the sub-unit increases during the ageing process. There is also a decline in the level of the total $O.D._{260}$ absorbing material on the gradients of 15 per cent in the first 12 hr and 26 per cent in 24 hr. It has been known for a number of years[9] that excised plant tissue incubated on water exhibits extensive losses in the levels of RNA. The data of Fig. 2 suggest that in pea tissue part of this loss occurs in the polysomes.

[9] A. J. TREWAVAS, *Progress in Phytochemistry*, in press.

Table 2 shows the data of three separate experiments in which batches of tissue were incubated in the presence and absence of IAA for 12 hr and the polysomes then isolated and separated. The results indicate that auxin has increased the level of the polysomes by

TABLE 2. THE EFFECT OF AUXIN ON THE LEVEL OF POLYSOMES AND MONOSOMES IN ETIOLATED PEA TISSUE

	Polysomes		Monosomes		"Sub-unit"	
	+IAA	−IAA	+IAA	−IAA	+IAA	−IAA
Expt. No.						
1	4·64	2·92	2·76	2·85	0·30	0·39
2	5·30	2·95	2·70	2·56	—*	—
3	4·71	3·10	2·79	2·65	0·34	0·40
Mean	4·88	2·99	2·75	2·69	0·32	0·39
% Increase due to IAA=	+63		+2·2		−18	

Total O.D.$_{260}$ material in auxin-treated tissue=7·95. Total O.D.$_{260}$ material in untreated tissue=6·07. Increase due to auxin=32%.

Batches of fifty epicotyl sections were incubated in the presence and absence of 8×10^{-5} M IAA for 12 hr at 25° in darkness. At the end of this time period the polysomes were isolated and separated on density gradients as described in the Methods Section.

* The post-mitochondrial supernatant in these two samples was layered over 7 ml 1 M sucrose instead of the customary 5 ml. This resulted in virtually complete loss of this material.

Figures in the table represent total O.D.$_{260}$ absorbing material running in the particular region denoted.

TABLE 3. THE EFFECT OF SUCROSE ON THE LEVELS OF POLYSOMES AND MONOSOMES IN ETIOLATED PEA TISSUE

	Polysomes		Monosomes		"Sub-unit"	
	+Sucrose	−Sucrose	+Sucrose	−Sucrose	+Sucrose	−Sucrose
Expt. No.						
1	4·27	2·59	1·86	2·13	0·56	0·80
2	4·06	2·74	1·93	2·25	0·73	1·07
Mean	4·16	2·67	1·89	2·19	0·64	0·93
% Increase due to sucrose=	+56		−13		−31	

Total O.D.$_{260}$ material in sucrose-treated sections=6·96. Total O.D.$_{260}$ material in untreated section =5·81. Increase due to sucrose=+15%.

Batches of forty epicotyl sections were incubated in the presence and absence of 2% sucrose for 12 hr at 25° in darkness. At the end of this time period the polysomes were isolated and separated on density gradients as described in the Methods Section.

Figures in the table represent total O.D.$_{260}$ absorbing material running in the particular region denoted.

+63 per cent, but that of the monosome has remained substantially constant and the level of the sub-unit has shown a slight decline. The total increase in O.D.$_{260}$ absorbing material on the gradients, +32 per cent, is in good agreement with the data of Table 1 which showed under the same conditions an increase in the post-mitochondrial RNA of +27 per cent.

Table 3 shows the effect of incubating stem sections in the presence and absence of sucrose for 12 hr. Sucrose, like auxin, produces an increase in the level of the polysomes but this time it appears to be partly at the expense of the monomer which shows a slight decrease. There is also an increase in the total level of RNA on the gradients of about +15 per cent.

If the data of Fig. 3 and Tables 2 and 3 are compared it is clear that both auxin and sucrose have only reduced the rate of decline of polysomes normally observed in excised tissue. The question then arises as to whether the rate of decline is reduced by an enhanced synthesis of polysomes or by a reduction in the rate of breakdown of the polysomes. Figure 1 shows that auxin enhances the synthesis of ribosomal RNA and thus it could be supposed that auxin has reduced the rate of loss of polysomes by increasing their rate of synthesis. To obtain further evidence on this point batches of stem sections were incubated in labelled orotic acid in the presence and absence of IAA for 2 hr (Table 4). The ribosomes were then

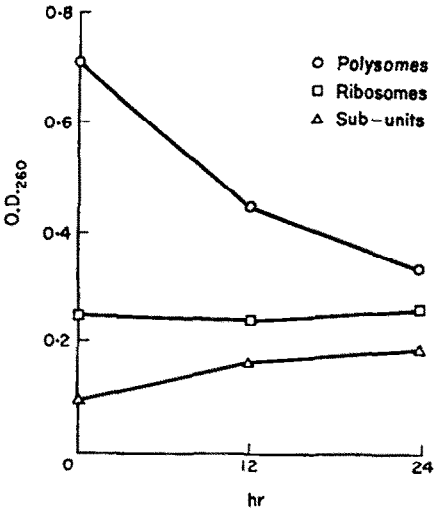

FIG. 3. EFFECT OF INCUBATING PEA-STEM SECTIONS ON LEVELS OF POLYSOMES.
Data from Figs. 2a–c by integrating the area under the appropriate peaks.

isolated and separated on density gradients. After fractionation, the specific activity of the three main fractions was determined. Auxin increases the uptake of orotic acid[1] into the tissue by about +25 per cent and this will have contributed to some extent to the increased labelling of both polysomes, monosomes and sub-unit. It can be seen, however, that the increase in the specific activity of the polysomes, +86 per cent, is considerably higher than that in the monosome, +47 per cent. These data support the notion that IAA may specifically enhance the level of polysomes in this tissue by increasing their rate of synthesis.

The problem remained as to whether the rate of synthesis of ribosomal protein was increased. In a previous paper we were able to show that auxin enhanced the incorporation of amino acids into protein in this tissue.[1] Furthermore, cell fractionation revealed that there was an auxin-increased labelling of all cell fractions to about the same degree. No attempt was made to remove nascent protein from the microsomal fractions and this may have interfered with the measurements of a specific effect of auxin on the synthesis of ribosomal protein. An attempt was therefore made to remove nascent and attached protein by: (*a*) employing label chase conditions, (*b*) employing conditions of high ionic strength, (*c*) sedi-

menting the ribosomes through strong sucrose, and (*d*) reprecipitating the ribosomes with streptomycin which results in ribosomes of high nucleic acid content[10] (Table 5). Batches of pea-stem sections were incubated in ^{3}H lysine and ^{14}C lysine for 1 hr and the isotope then removed and chased with cold lysine in the presence and absence of IAA. Ribosomes were then prepared as described in the Methods Section and reprecipitated with streptomycin. The ratio of the $^{14}C/^{3}H$ was then determined in both the supernatant and the ribosomal

TABLE 4. THE EFFECT OF IAA ON THE INCORPORATION OF LABELLED OROTIC ACID INTO THE POLYSOMES OF ETIOLATED PEA TISSUE

	Polysomes		Monosomes		"Sub-unit"	
	+IAA	−IAA	+IAA	−IAA	+IAA	−IAA
	112*	61	78	53	80	49
% Increase due to IAA	+86		+47		+65	

* Figures represent the specific activity in terms of disintegrations/min μg RNA in the particular fractions denoted.

Batches of fifty epicotyl sections were incubated in 200 μc 5-[^{3}H]-orotic acid in the presence and absence of 8×10^{-5} M IAA for 2 hr at 25°. The polysomes were isolated as described in the Methods Section and separated on sucrose gradients. After fractionation the RNA, together with 1 mg carrier, was precipitated onto millipore filters with 5% trichloroacetic acid. The filters were dried and counted after submersion in 15 ml toluene scintillator solution. Corrections for self-absorption were made from standard curves prepared using labelled pea nucleic acid.[17]

TABLE 5. THE EFFECT OF IAA ON THE SYNTHESIS OF RIBOSOMAL PROTEIN IN ETIOLATED PEA-STEM TISSUE

Ribosomal protein			Supernatant protein		
^{3}H dpm	^{14}C dpm	$^{14}C/^{3}H$	^{3}H dpm	^{14}C dpm	$^{14}C/^{3}H$
17,700	15,000	1·07	84×10^{5}	104×10^{5}	1·24

Batches of twenty-five epicotyl sections were incubated in 50 μc [^{3}H]-lysine and 5 μc [^{14}C]-lysine for 1 hr. The sections were thoroughly washed with 10^{-3} M L-lysine end then incubated in 10^{-3} M lysine ± IAA (5×10^{-5} M) for a further 2 hr. At the and of this time period the two batches were bulked, homogenized using 0·6 M NH_4Cl as described in the Methods Section and the polysomes prepared. After spinning the supernatant was carefully removed with a syringe and the inside of the tube cleaned with tissue paper. The pellet was resuspended in 1 ml 5 per cent sucrose and precipitated with 0·15 ml 2 per cent streptomycin sulphate. Both ribosomal protein and supernatant protein were freed of any attached amino acid by solution and reprecipitation with acid and alkali as previously described.[1]

protein. It can be seen that auxin does not increase the $^{14}C/^{3}H$ ratio, implying the absence of any specific effect of auxin on the synthesis of ribosomal protein. This experiment has been repeated using different levels of labelled precursors and two cycles of precipitation by streptomycin. This failed however to give results different from those in Table 5. The $^{14}C/^{3}H$ of the ribosomal protein was 0·67 and that of the supernatant protein 0·62.

[10] M. L. PETERMANN, *The Physical and Chemical Properties of Ribosomes*, pp. 43—44. Elsevier, New York (1964).

DISCUSSION

It has now been shown that auxin specifically alters the pattern of protein synthesis and increases the rate of incorporation of amino acids into protein in etiolated pea-stem tissue.[1,11] Table 1, however, shows that there is no overall increase in the level of protein after 12 hr incubation in IAA. The apparent dichotomy in these data cannot be resolved at the present time. The data of Table 2 indicates that it is unlikely to be a result of the limitation of the level of polysomes. The answer might lie in the protein precursor pools of amino acids. These show a very rapid depletion in excised tissue[12] and might provide a more fundamental limitation on the rate of protein synthesis than the levels of polysomes.

Auxin appears to increase the levels of polysomes in pea tissue by altering the rate of synthesis of nucleic acid. Interestingly enough it appears to do this by altering the rate of synthesis of ribosomal—rather than messenger—RNA. We have as yet no evidence on the mechanism by which sucrose increases the level of polysomes. It would seem unlikely that it would do so by altering specifically the synthesis of ribosomal RNA. Sucrose may act in this system by providing higher levels of respirable substrate and thereby allowing a more rapid rate of nucleic acid synthesis. It has been noted that incubation of oat coleoptile sections in sucrose solutions results in a much higher rate of turnover of the adenine nucleotides[13,14] and it seems not unreasonable to suppose that this could occur in pea tissue.

Since auxins are plant-growth hormones it could be concluded that there is a general relationship between growth in plants and the level of polysomes. This could also be a conclusion from the data given in Table 3. Sucrose enhances the rate of growth of excised pea tissue[7] and increases the level of polysomes. Other data is in agreement with this conclusion. Etiolated pea-stem tissue shows a rapid decline in the rate of growth in response to exposure to white light. Consonant with this decline in growth the levels of polysomes show a drop of 40–50 per cent. (A. J. Trewavas, unpublished data.)

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* var. Alaska) were obtained at regular intervals from Daniels, Norwich, and were stored at 2°. 5-[^{3}H]-Orotic acid (4 c/mM) U-[^{14}C]-leucine (150 mc/mM), 4,5-[^{3}H]-L-leucine, (G) [^{3}H]-adenosine (3·45 c/mM) and (U) [^{14}C]-adenosine (350 mc/mM) were obtained from the Radiochemical Centre, Amersham.

20 mm sections of the third internodes of dark grown epicotyls were obtained as described by Christiansen and Thimann[15] with the modification that a 3 mm tip was removed. RNA and protein and the specific activity of protein were determined as previously described.[1] Labelled nucleic acids from pea stems were prepared by a sodium lauryl sulphate/phenol method similar to that described by Cherry and Chroboczek.[16] Phenol was removed by diethyl ether and the ether removed with N_2.

The labelled nucleic acids were separated on a methylated albumin-kieselguhr column,[2] using an 0·35 M NaCl to 1·0 M NaCl gradient with a total eluting volume of 800 ml. 3-ml fractions were collected. The labelled nucleic acids were counted by a new technique which

[11] B. D. PATTERSON and A. J. TREWAVAS, *Plant Physiol.* **42**, 1081 (1967).
[12] J. H. HOLLEMAN and J. L. KEY, *Plant Physiol.* **42**, 29 (1967).
[13] A. J. TREWAVAS, Ph.D. Thesis, University of London (1965).
[14] A. J. TREWAVAS, I. R. JOHNSTON and E. M. CROOK, *Biochem. Biophys. Acta* **136**, 301 (1967).
[15] G. S. CHRISTIANSEN and K. V. THIMANN, *Arch. Biochem.* **26**, 230 (1950).
[16] J. H. CHERRY and H. CHROBOCZEK, *Phytochem.* **5**, 411 (1966).

is described in greater detail elsewhere.[17] The technique overcomes the problem of self-absorption by dissolving the cetyltrimethylammonium salt of the nucleic acids in toluene scintillator solution. Each 3-ml fraction from the column was diluted with 10 ml 0·01 M NaCl/0·001 M Na phosphate, pH 6·7, containing 1 mg yeast carrier RNA. 1 ml 1 per cent cetyltrimethylammonium bromide was added and the tube mixed and allowed to stand overnight before filtration onto a Whatman GF/C pre-cut glass-fibre filter. The filters were allowed to dry overnight at room temperature and were then boiled for 30 sec in 1 ml 2-methoxyethanol in a scintillation vial before addition of 15 ml toluene scintillator (4 g PPO, 0·04 g dimethyl POPOP/l. toluene). The vials were counted in a Packard Tricarb scintillation counter using automatic external standardization and quench curves prepared with chloroform.

ISOLATION AND SEPARATION OF POLYSOMES

Batches of fifty epicotyl sections were chilled after harvesting by incubation for 5 min in ice-cold water and then blotted dry. They were homogenized in 3 ml 12 per cent sucrose, 0·52 M NH_4Cl, 0·005 M $MgCl_2$, 0·01 M Tris, pH 7·5, by pressing the tissue with a pestle in a mortar. The homogenate was filtered through cheese-cloth and spun at 14,000 *g* for 10 min. The supernatant was made 0·1 per cent in sodium deoxycholate and layered with a wide-bore pasteur pipette onto 5 ml 35 per cent sucrose, 0·1 M NH_4Cl, 0·01 M Tris, pH 7·4, 0·005 M $MgCl_2$ and spun in the Spinco Ti 50 head for 2 hr at 50,000 rev/min. The nearly transparent pellets were resuspended by gently stroking the precipitate with a rounded glass rod in 0·2 ml 5 per cent sucrose, 0·005 M $MgCl_2$, 0·01 M Tris, pH 7·4, 0·1 M NH_4Cl. The suspension was then transferred with a wide-bore pasteur pipette onto a 27 ml 10 to 35 per cent exponential sucrose gradient containing 0·1 M NH_4Cl, 0·01 M Tris, pH 7·4, 0·005 M $MgCl_2$. The tube was filled with liquid paraffin and the gradient spun in the SW 25 at 25,000 rev/min for 80 to 90 min. The head was allowed to come to rest with the brake off and the gradient fractionated, after insertion of a capillary to the bottom of the gradient, with the aid of a peristaltic pump and a drop counter. 0·75-ml fractions were collected and the $O.D._{260}$ read with the aid of micro cells. All of the above operations were carried out as near 0° as possible.

[17] A. J. TREWAVAS, *Anal. Biochem.*, in press.