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RIBONUCLEIC ACID METABOLISM AND CELL EXPANSION IN OAT COLEOPTILE

YOSHIO MASUDA[1], GEORGE SETTERFIELD AND S. T. BAYLEY

Department of Biology, Carleton University, Ottawa, Canada
and
Division of Biosciences, National Research Council, Ottawa, Canada

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RNA metabolism in oat coleoptiles was studied using physiological responses to 5-FU and actinomycin D; autoradiographic detection of RNA and protein synthesis; and estimation of ribosomal concentration by analytical ultracentrifugation. 5-FU failed to inhibit growth of either intact coleoptiles or isolated coleoptile segments but completely blocked cell division in roots. Actinomycin D markedly inhibited auxin-induced expansion of coleoptile segments. When supplied to isolated segments from coleoptiles of various lengths the RNA precursors cytidine, adenine and adenosine all showed weak incorporation into RNA of nuclei and in some cases, to a lesser extent, RNA of cytoplasm. IAA did not affect this RNA synthesis but it was considerably reduced by actinomycin D. A proportion of the label incorporated from RNA precursors was not removable with either RNase, PCA or hot TCA but was extracted by trypsin. The amount of this spurious incorporation increased with coleoptile age, as did the ability to incorporate labelled amino acids. The concentration of both free and bound ribosomes does not increase in growing coleoptiles and may even decline. Free ribosomes decline markedly in fully grown coleoptiles while the proportion of bound ribosomes increases. It is concluded that young coleoptiles contain a full complement of ribosomes necessary for subsequent growth but normal growth is dependent on continued production of an actinomycin D-sensitive messenger-type RNA. No evidence for auxin mediation of RNA synthesis was found.

Both the metabolic processes underlying plant cell expansion and the specific point of action of auxins in promoting these processes remain poorly understood. On the basis of rather indirect evidence, several authors have suggested that RNA may be directly involved in the expansion process (*1–8*).

Abbreviations: DNA, deoxyribonucleic acid; DNase, deoxyribonuclease; DOC, sodium deoxycholate; 5-FU, 5-fluorouracil; IAA, indole-3-acetic acid; PCA, perchloric acid; RNA, ribonucleic acid; RNase, ribonuclease; TCA, trichloroacetic acid.

[1] Present address: Laboratory of Cell Biology, Faculty of Science, Osaka City University, Sugimoto-cho, Sumiyoshi-ku, Osaka, Japan.

The central role of RNA in protein synthesis makes this suggestion reasonable but as yet little data on the behaviour and function of RNA in expanding plant cells are available (*9*).

The study reported here is an attempt to learn RNA metabolism in a classical object for cell expansion studies, the oat coleoptile. The approaches used were physiological studies with a pyrimidine analog, 5-fluorouracil, and an inhibitor of DNA dependent RNA synthesis, actinomycin D; autoradiographic studies using labelled RNA and protein precursors; and estimation of ribosomal concentrations through sedimentation analysis. In general the results indicate that in the oat coleoptile the bulk of RNA is formed before the grand period of cell expansion, and that there is very little RNA turnover subsequently. Synthesis of a small amount of RNA which does occur during cell expansion may, however, be essential for the growth process. No evidence for a stimulation of RNA synthesis by auxin was obtained.

MATERIAL AND METHODS

Plant material

The plants used were seedlings of *Avena sativa*, varieties Victory and Lanark. Seedlings were germinated in the dark at 25°, as described previously (*1*). Growth of coleoptiles and roots of intact seedlings was measured with a ruler under red light and cell length and cell number were determined by the method of BURSTRÖM (*10*). For studies on excised segments of coleoptiles, seedlings were grown in darkness until the coleoptiles were of desired length (10 to 50 mm), the apical 3 mm was decapitated, and after 2 hours a segment 5 mm long was cut from the upper region of the coleoptile with a double-bladed tool. For growth studies ten such segments were placed on 5 ml of the solution to be tested and growth was measured with an ocular micrometer in a dissecting microscope, using red light.

Autoradiography

The following tritiated compounds were used: cytidine (1.3 c/mM), adenine hydrochloride (2.16 c/mM), adenosine (0.475 c/mM), DL-leucine hydrochloride (5.45 c/mM), and proline (1.0 c/mM). Proline was obtained from Schwarz Bioresearch, Inc., Mount Vernon, N.Y. and the other compounds from New England Nuclear Corp., Boston, Mass. To supply isotope to the tissues, groups of four coleoptile segments were floated for 5 hours on 2 ml of solution containing 5 μc/ml labelled precursor, along with other additions such as IAA and glucose, as noted in the RESULTS section.

Following incubation the segments were fixed in ethanol-acetic acid (3:1), dehydrated, embedded in paraffin, and sectioned longitudinally at 8 μ thickness. Sections were mounted on subbed slides, deparaffined and hy-

drated. At this stage various extraction procedures were applied: 2% PCA for 20 min at 4° to remove soluble nucleotides (*11*); 0.04% RNase (Worthington Biochemical Corp.) for 2 hr at 37° to remove RNA (*12*), followed by 2% PCA at 4° for 20 min; 10% PCA for 2 hr at room temperature to remove RNA (*11*); 0.04% DNase (Worthington Biochemical Corp.) in 0.003 M $MgSO_4$ for 2 hr at 37° to remove DNA (*12*); 5% TCA for 15 min at 90° to remove nucleic acids (*12*); 1% trypsin, pH 8.0, at 32–35° overnight to partially remove protein. Following extraction the slides were washed several times in water, dehydrated, dipped in 0.3% parlodion, and dried for 24 hr. The slides were then coated with Kodak NTB-3 emulsion and exposed at 4° for 1–3 weeks (*13*). The autoradiographs were developed in Kodak D19 for 10 min at 4°, rinsed, fixed for 20 min, dehydrated in cold absolute ethanol, cleared in xylol and mounted in oil of R.I. 1.460. Slides were viewed alternately by phase contrast and bright-field illumination and frequency of silver grains per square micron determined. Background counts of silver grains in the area adjacent to the sections were subtracted from all values. The data are presented as mean values of grain counts over 10 nuclei and 10 cytoplasmic regions in each of three different coleoptiles.

Cell fractionation and analysis of ribosomal components

a) Estimation of free ribosomes—In each experiment, 250 coleoptiles of the same age were harvested, their lengths recorded and averaged, the primary leaves removed and the coleoptiles cooled on ice. The fractionation procedure, which was essentially that of Ts'o et al. (*14*), was carried out in the cold. The coleoptiles were ground with a pestle and mortar in aqueous 0.4 M sucrose, 0.5–1.0 ml per g wet tissue. The bulk of the wall material was removed from the homogenate by centrifuging at 12,000×*g* for 10 min; occasionally with older tissues, this centrifugation was repeated. Larger cell debris, nuclei and mitochondria were removed by sedimenting at 40,000×*g* for 10 min. The microsomal fraction was then obtained as a pellet from the resulting supernatant by centrifuging at 105,000×*g* for 75 min. This pellet was redispersed in a small volume of water by gentle stirring for 1 hr. The suspension obtained was centrifuged for 10 min at 15,000×*g* and the supernatant made up to a final volume of 1 ml. With a delay of never more than a few hours, this final suspension was analysed in a Spinco model E ultracentrifuge at 20° with Schlieren optics and a bar angle of 60°.

Relative concentrations of sedimenting components were estimated from the areas of the significant peaks above the base lines of the sedimentation patterns. The same refractive index increment was assumed for all sedimenting components. To compare the relative concentrations of components in different experiments, the exposure times were chosen so that the principal sedimenting component, the 78–80 S peak, occupied roughly the same position in the cell. For this reason and because of the errors inherent in the present work, it was thought unnecessary to make radial corrections.

b) Estimation of ribosomes bound to membranes—The fractionating procedure was similar to that just described. Five hundred coleoptiles, less primary leaves, from each of several different ages, were ground in 0.4 M sucrose with 5×10^{-3} M Tris buffer, pH 7.2, and 5×10^{-4} M $MgSO_4$. The cell homogenate was fractionated according to the scheme described above, except that in most cases, the centrifugation at $40{,}000\times g$ was omitted so that the final pellet contained all the membranous components from the cell. The final pellet was redispersed in a small quantity of the same buffer with $MgSO_4$ as before, and made up to a final volume of 1 ml. Immediately before analysis in the ultracentrifuge, this final 1 ml of suspension was divided in two. To one half, 0.5 ml of 0.6% DOC in buffer containing $MgSO_4$ was added, while the control received 0.5 ml of the buffer with $MgSO_4$. The two samples were run simultaneously in the analytical ultracentrifuge using a wedge cell. The relative concentrations of the sedimenting components were estimated as before.

RESULTS

Growth studies

Oat seeds were imbibed on damp filter paper for 24 hr at 25° and then groups of 20 seeds were transferred to 10 ml of water containing various concentrations of 5-FU. Fig. 1 shows the course of elongation of the intact

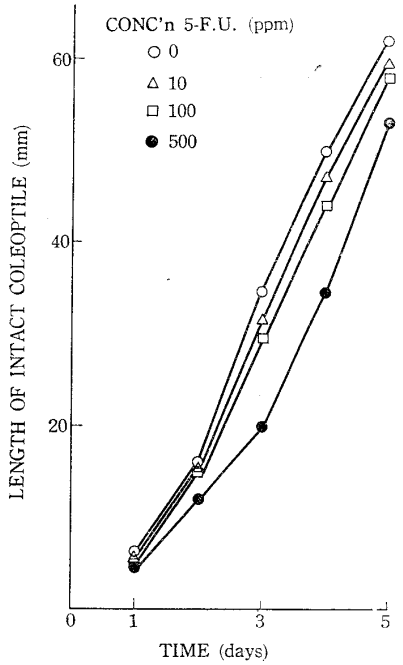

Fig. 1. Effect of increasing concentrations of 5-FU on the elongation of intact coleoptiles. Seeds were imbibed on water for one day and then transferred to 5-FU solutions. Points represent mean lengths from 20 seedlings.

coleoptiles of these seedlings. Clearly, elongation of the coleoptiles was quite insensitive to 5-FU supplied via the roots, since even 500 ppm 5-FU

gave only a slight reduction in length. Fig. 2 shows the results of a similar experiment with intact seedlings in which both coleoptile length and root length were measured after 6 days treatment with various concentrations of 5-FU. Again, coleoptile length was essentially unaffected by 5-FU, whereas the growth of roots was strongly suppressed by even 100 ppm 5-FU. An analysis of cell length and number in these plant organs is given in Table I. As expected from the data on coleoptile length, 5-FU affected neither cell length nor cell number in the coleoptiles. On the other hand, in the root cell division was markedly inhibited while the average cell length was, if anything, slightly stimulated by the 5-FU.

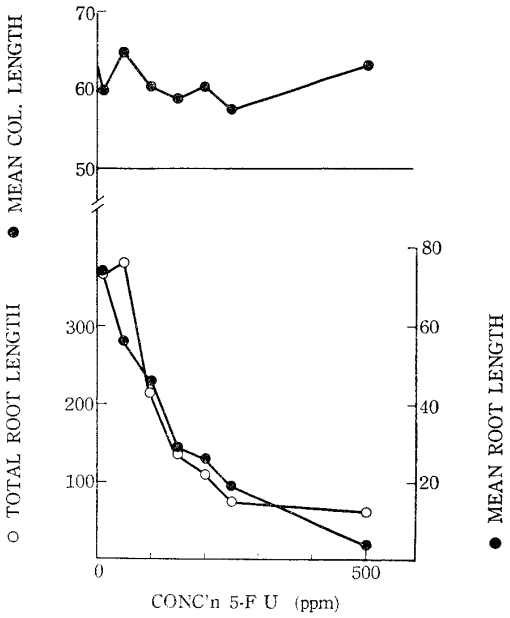

Fig. 2. Effect of increasing concentrations of 5-FU on the growth of coleoptiles and roots of intact oat seedlings. Seeds were imbibed on water for one day and then transferred to 5-FU solutions. Mean lengths of 20 seedlings after 6 days treatment with 5-FU are shown.

TABLE I

Effect of 5-FU on cell length and cell number in coleoptiles and roots of intact oat seedlings

Twenty seedlings, one day old, were placed in 20 ml of 500 ppm 5-FU. Measurements were made after 6 days treatment.

	Coleoptile (parenchyma)		Roots (epidermis)	
	Water	5-FU	Water	5-FU
Organ length(mm)	63±0.94[a]	62.9±1.82	73.4±4.34	14.4±0.96
Mean cell length(μ)	335±10.4	332±11.4	307±12.3	348±11.7
Mean cell number	188.1	189.9	237.8	12.9

[a]In all Tables ± values represent the standard error of the mean.

These results suggest that 5-FU has little effect on the process of cell expansion but strongly inhibits cell division. However, cell division does occur in young coleoptiles and 5-FU might be expected to give a reduction in coleoptile length through interference with this process. To circumvent the possibility that 5-FU was not being transported to the coleoptile in the intact seedling, excised segments from coleoptiles 16–18 mm long were treated with 500 ppm 5-FU in the presence and absence of IAA. The results shown in Fig. 3 again indicate that this high concentration of 5-FU had no effect on cell expansion. Similar results were obtained with segments isolated from 10 mm long coleoptiles.

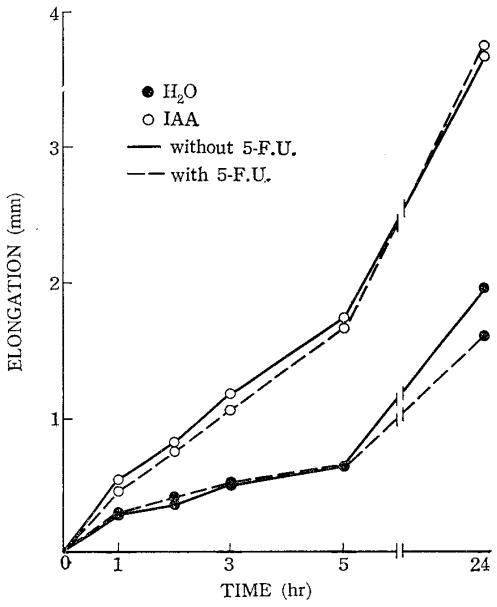

Fig. 3. Effect of 500 ppm 5-FU on the elongation of 5 mm coleoptile segments excised from 16–18 mm coleoptiles. IAA concentration 8 ppm. Points represent means from ten segments.

Excised coleoptile segments were also treated with actinomycin D at various concentrations, in the presence and absence of IAA, and expansion growth followed. Fig. 4 indicates that actinomycin D markedly inhibited the stimulation of cell expansion produced by IAA, the inhibition being quite noticeable at 10 ppm of the antibiotic. Expansion of the controls lacking IAA was unaffected by actinomycin D. These results are very similar to those of NOODÉN and THIMANN (7).

Autoradiographic studies

In order to measure the sites and relative amounts of RNA synthesis in excised coleoptile segments, a series of autoradiographic studies using tritium-labelled RNA precursors were performed. Since it was possible that RNA metabolism varied with age (length) of coleoptile, the segments were cut from coleoptiles of various lengths. Fig. 5 shows the results of a preliminary experiment in which the growth potentials of 5 mm segments from coleoptiles 10, 20, 30 and 50 mm in length were compared.

Segments from the younger coleoptiles were capable of considerable expansion in 5 hr, when supplied with IAA, while segments from 50 mm coleoptiles were essentially incapable of further expansion.

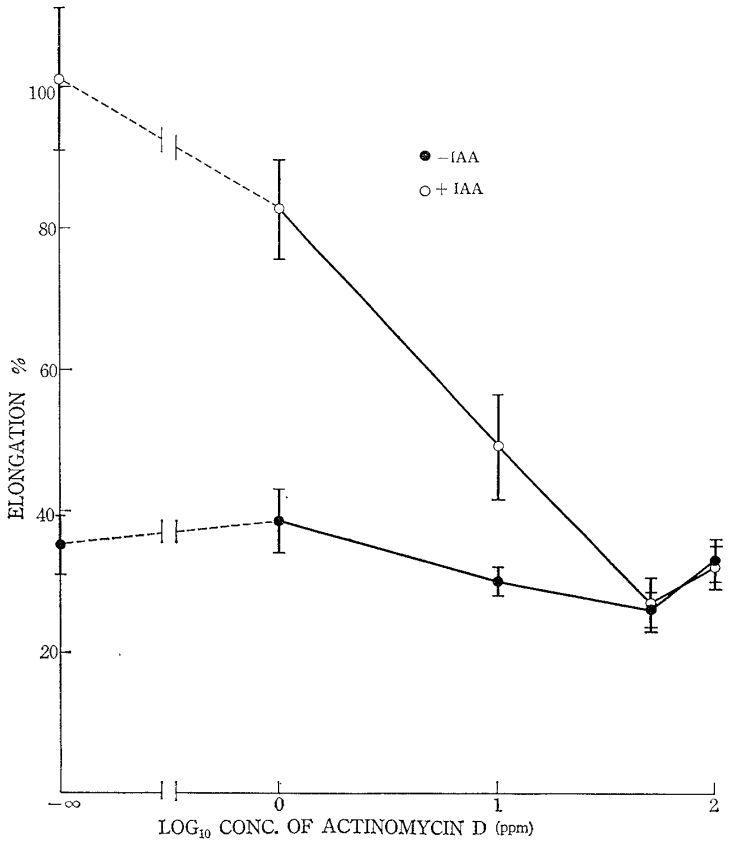

Fig. 4. Effect of increasing concentrations of actinomycin D on elongation of 5 mm coleoptile segments excised from 30 mm coleoptiles. IAA concentration 8 ppm. 2% sucrose was included in all treatments. Points represent means from 8 segments after 20 hr treatment. Vertical bars indicate standard errors.

The incorporation of radioactive label from ^{3}H-cytidine by segments comparable to those above is shown in Fig. 6. Radioactivity is expressed as mean silver grains per square micron, as determined in autoradiographs. As can be seen by the solid curves, measurable uptake of tritium label occurred, and the amount increased with age of coleoptiles from which the segments were removed. However, this label did not show a typical RNA labelling pattern (*4, 12*), in which the nucleolus is more heavily labelled than the chromatin region of the nucleus. Further, the label was only partially removed from the nucleus by RNase treatment and, except for the youngest segments (10 mm coleoptile), was not significantly extracted from cytoplasm by this treatment.

It should be pointed out that the density of grains in these preparations

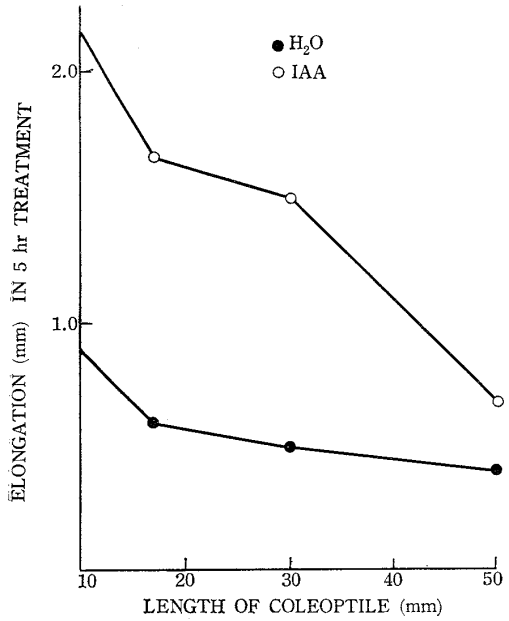

Fig. 5. Elongation of excised coleoptile segments, taken from coleoptiles of various lengths, incubated with and without 8 ppm IAA for 5 hr. Points represent means from ten segments.

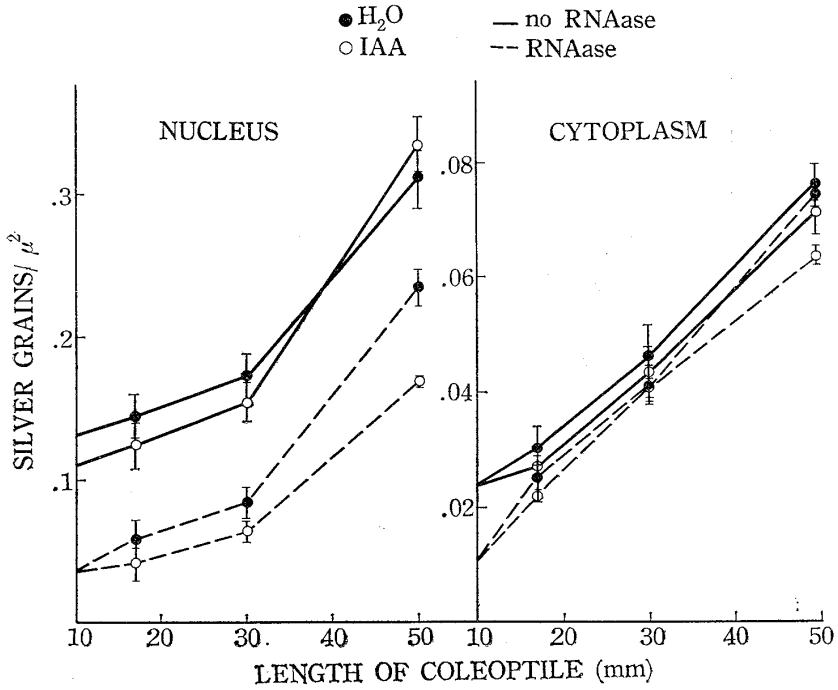

Fig. 6. Frequency of silver grains (grains/μ^2) in autoradiographs of coleoptile cells supplied with ^{3}H-cytidine. Excised coleoptile cylinders from coleoptiles of various lengths (abscissa) were incubated 5 hr on 5 μc/ml ^{3}H-cytidine, with (○) and without (●) 8 ppm IAA. Segments were treated with either cold PCA (solid lines) or RNase and cold PCA (broken lines) prior to autoradiography. Autoradiographs exposed for 2 weeks. Vertical bars indicate standard errors.

is about an order of magnitude lower than is found in autoradiographs of tissues active in RNA synthesis such as root tips (*11*) or freshly-cut artichoke tuber tissue (*4*), when comparable autoradiographic procedures are used. Thus, Fig. 6 indicates, by comparison of the solid and broken curves, that only a small incorporation of ^{3}H-cytidine into nuclear RNA took place and almost no labelled RNA was found in the cytoplasm. Further, the results indicate that IAA had little or no effect on incorporation of ^{3}H-cytidine into RNA as measured by this technique.

To test further the effect of IAA on RNA synthesis, segments from 30 mm long coleoptiles were supplied with ^{3}H-cytidine in the presence and absence of IAA and glucose. The resultant silver grain counts, shown in Table II, are similar to those of the previous experiment (Fig. 5). Again

TABLE II

Frequency of silver grains (grains/μ^2) in autoradiographs of coleoptile cells supplied with ^{3}H-cytidine for 5 hr in the presence and absence of 8 ppm IAA and 2% glucose

5 mm segments from coleoptiles 30 mm long were used. Autoradiographs exposed for 2 weeks.

		Treatment			
Cell area	Extraction after fixation	Water	Glucose	IAA	IAA+ glucose
Nucleus	PCA, 4°	0.239±.006	0.216±.005	0.234±.011	0.253±.012
	RNase+ PCA, 4°	0.156±.008	0.091±.009	0.138±.004	0.086±.001
Cytoplasm	PCA, 4°	0.048±.003	0.054±.002	0.055±.004	0.054±.002
	RNase+ PCA, 4°	0.050±.000	0.048±.005	0.051±.001	0.048±.002
Mean segment length after 5 hr(mm)		5.5	5.5	6.6	6.6

the heavier incorporation of isotope was over the nuclei and this label was only partially removed by RNase. The lighter incorporation in the cytoplasm was essentially unaffected by RNase. Neither IAA nor glucose had any significant effect on the total incorporation into either nucleus or cytoplasm and IAA did not change the proportion of nuclear label extractable by RNase. Glucose, on the other hand, both with and without IAA, did significantly increase the amount of RNase-extractable label in the nuclei. The nature of this change in nuclear label is obscure but it was not correlated with IAA stimulated expansion. Again, it must be emphasized that the grain counts in these experiments were relatively low and the change in nuclear labelling due to glucose treatment represents a very small, although not necessarily unimportant, amount of RNA.

Since in these experiments a considerable amount of the isotope incor-

porated during treatment with ^{3}H-cytidine was resistant to RNase extraction, an experiment was performed to compare the efficiency of the enzyme with chemical methods of extraction of RNA. Sections from the coleoptile segments given water treatment in the previous experiment were extracted prior to autoradiography with either cold PCA, RNase and cold PCA, PCA at room temperature, hot TCA or trypsin. As shown in Table III, room temperature PCA and hot TCA were only slightly more effective than RNase in removing label from the nucleus, and like RNase were inactive towards the cytoplasmic label. Only trypsin gave a marked reduction in label below the RNase level, in both nuclei and cytoplasm. It was concluded from this experiment that the great majority of the label remaining after RNase treatment was definitely not in RNA but rather in some insoluble material associated with protein.

TABLE III

Frequency of silver grains (grains/μ^2) in autoradiographs of coleoptile cells supplied with ^{3}H-cytidine and extracted in various ways, after fixation

Conditions as for the water treatment in Table II.

Extraction	Cell area	
	Nucleus	Cytoplasm
PCA, 4°	0.239±.002	0.048±.003
RNase+PCA, 4°	0.156±.008	0.050±.000
PCA, 20°	0.139±.016	0.052±.003
TCA, 90°	0.130±.001	0.051±.003
Trypsin	0.015±.000	0.010±.001

In an effort to avoid the residual isotope incorporation encountered with cytidine as an RNA precursor, ^{3}H-adenine and ^{3}H-adenosine were fed to coleoptile segments. The results of grain counting in this material are presented in Tables IV and V and show marked similarity to the results with ^{3}H-cytidine. The total incorporation of tritium rose with age of coleoptile, and a part of the nuclear, and almost all of the cytoplasmic, incorporation were resistant to RNase. The nuclear label removable by RNase again indicated a small synthesis of RNA in this organelle. The magnitude of the incorporation of both precursors into nuclear RNA varied, with the segments from older coleoptiles showing a greater proportion of label extractable by RNase. With both precursors there was some suggestion of RNase-extractable label in the cytoplasm, particularly in the segments from 30 mm coleoptiles. However, the difference in cytoplasmic silver grain density between the extracted and unextracted tissues was close to the limit of sensitivity of the autoradiography.

Since actinomycin D inhibited coleoptile growth and is presumed to inhibit RNA synthesis, an experiment was performed to test the effect of

TABLE IV

Frequency of silver grains (grains/μ^2) in autoradiographs of coleoptile cells supplied with ^3H-adenine for 5 hr

5 mm segments from coleoptiles 10, 30 and 50 mm long were used. Autoradiographs exposed for 1 week.

Cell area	Extraction after fixation	Coleoptile length-mm		
		10	30	50
Nucleus	PCA, 4°	0.296±.035	0.460±.010	0.595±.018
	RNase+PCA, 4°	0.054±.010	0.115±.009	0.164±.022
Cytoplasm	PCA, 4°	0.069±.006	0.137±.005	0.149±.002
	RNase+PCA, 4°	0.041±.002	0.090±.002	0.128±.004

TABLE V

Frequency of silver grains (grains/μ^2) in autoradiographs of coleoptile cells supplied with ^3H-adenosine

Feeding conditions as in Table IV. Autoradiographs exposed for 3 weeks.

Cell area	Extraction after fixation	Coleoptile length-mm		
		10	30	50
Nucleus	PCA, 4°	0.158±.008	0.560±.005	0.748±.040
	RNase+PCA, 4°	0.053±.009	0.146±.007	0.417±.055
Cytoplasm	PCA, 4°	0.044±.003	0.173±.020	0.433±.006
	RNase+PCA, 4°	0.045±.002	0.123±.003	0.444±.044

actinomycin D on incorporation of ^3H-cytidine. Ten ppm actinomycin D, which markedly inhibited cell expansion (Fig. 4), was supplied to cells in the presence of IAA and glucose. In order to increase the chances of detecting differences in labelled RNA the time of treatment with cytidine was extended from the usual 5 hr to 24 hr. The results of grain counts (Table VI) over the actinomycin-treated and control cells clearly show that actinomycin D markedly reduced the amount of RNase-extractable tritium incorporated into the nuclei. Furthermore, with the longer cytidine treatment time used in this experiment a significant cytoplasmic label, which was RNase-extractable, was detected in the control cells and this label was also reduced by actinomycin D. Clearly actinomycin D at 10 ppm severely inhibited the RNA synthesis occurring in expanding coleoptile cells.

Since all three nucleic acid precursors were partially metabolized to non-nucleic acid materials, and since trypsin was the only agent found effective in removing this label, it seemed possible that protein metabolism might be varying with age of the coleoptiles. To investigate this, two autoradiographic experiments were performed using ^3H-leucine and ^3H-proline as labelled precursors. Grain counts over the cells supplied with leucine are given in Table VII. In the material supplied with proline the label was too

TABLE VI

Frequency of silver grains (grains/μ^2) in autoradiographs of coleoptile cells supplied with ^{3}H-cytidine for 24 hr in the presence of 10 ppm IAA and 2% glucose, without and with 10 ppm actinomycin D

5 mm segments from coleoptiles 25 mm long were used. Autoradiographs exposed for 1 week.

Cell area	Extraction after fixation	Treatment	
		IAA+glucose	IAA+glucose+act. D
Nucleus	PCA, 4°	0.846±.110	0.298±.049
	RNase+PCA, 4°	0.148±.010	0.108±.016
Cytoplasm	PCA, 4°	0.183±.016	0.063±.013
	RNase+PCA, 4°	0.080±.012	0.047±.012

heavy for direct grain counting, and grain density was estimated photometrically (Table VIII). With leucine, there was a light label over both nuclei and cytoplasm and the amount of label increased in segments from older coleoptiles. This label, as expected, was unaffected by RNase and DNase but significantly lowered by trypsin digestion. Proline also showed increased incorporation in the older segments. The incorporation of these amino acids, then, parallels quite closely the incorporation of the label of the nucleic acid precursors into material resistant to RNase. It seems a reasonable possibility that the non-RNA label found after supplying nucleic acid precursors represents metabolism of these compounds to protein precursors and subsequent incorporation into protein.

TABLE VII

Frequency of silver grains (grains/μ^2) in autoradiographs of coleoptile cells supplied with ^{3}H-leucine for 5 hr.

5 mm segments isolated from coleoptiles 20, 30 and 50 mm long were used. Autoradiographs exposed for two weeks.

Cell area	Extraction after fixation	Coleoptile length-mm		
		20	30	50
Nucleus	PCA, 4°	0.035±.005	0.045±.004	0.063±.007
	RNase+DNase	0.040±.002	0.048±.003	0.047±.003
	Trypsin	0.014±.003	0.013±.001	0.018±.002
Cytoplasm	PCA, 4°	0.022±.004	0.050±.004	0.054±.003
	RNase+DNase	0.019±.003	0.048±.003	0.058±.002
	Trypsin	0.011±.001	0.009±.001	0.018±.001

TABLE VIII

Frequency of silver grains in arbitrary units determined with a densitometer, in coleoptile cells supplied with ^{3}H-proline

Feeding conditions as in Table VI. Autoradiographs exposed for 2 weeks.

Extraction after fixation	Coleoptile length-mm		
	20	30	50
PCA, 4°	3.7±0.37	9.1±0.36	10.1±1.27

Studies on ribosomal concentration

The concentration of free ribosomes (i.e. ribosomes free of membranes following homogenization in sucrose) in coleoptiles ranging in length from 3 to 50 mm, as estimated from the total areas under the peaks in their sedimentation patterns, are given in Table IX. In all cases the 78–80S component was predominant. Components of about 118S and heavier were assumed to represent polyribosomes (*15*), while particles of about 60S, 40S and 20S were probably ribosomal subunits. Except in the shortest length coleoptiles studied (3 mm), coleoptile cells undergo few divisions, and the cell number per coleoptile remains constant. Since the same number of coleoptiles was used for each sedimentation pattern, the areas measured are directly proportional to the concentration of ribosomes per cell.

TABLE IX

Concentration of freely sedimenting ribosomes in coleoptiles as a function of age

Each determination was performed on 250 coleoptiles. Ribosomal concentrations are given in arbitrary units of area beneath significant ribosomal peaks in the analytical ultracentrifuge.

Coleoptile		Ribosomal concentration (area-arbitrary units)[a]
Age (days)	Length (mm)	
1.5–2	3	72
1.5–2	3–4	61
2.5	10	47
3	15	41
3	17	57
4	27	43
4.5	37	38
5–6	54	5
7	50	—

[a] Mean sedimentation coefficient of predominant peak=78S; other peaks at 118S, 60S, 40S, 20S.

As can be seen in Table IX, there was considerable random variation in the estimates of concentrations, probably mainly due to variable losses during fractionation. Nevertheless, it is clear that no significant increase in ribosomes occurred during cell expansion; rather, the concentration of ribosomes remained roughly constant or even possibly declined during the growth period. A sharp drop in concentration of ribosomes occurred when elongation ceased (50 mm).

The estimates of ribosomes presumably concern mainly ribosomes which are either free of or only loosely bound to the endoplasmic reticulum *in situ*. To study possible changes in concentration of membrane bound ribosomes a series of analyses was performed in which fractions were treated with DOC to disperse membranes and to free tightly bound ribosomes. The results of these analyses are summarized in Table X. In most of the experiments the whole homogenate, after a low speed centrifugation ($12{,}000\times g$ for 10 min) to remove wall material, was treated with DOC. In two cases, however, a microsomal fraction prepared after a cleaning spin of $40{,}000\times g$ was treated. Little difference was found between these two procedures. The only noticeable effect of the DOC on the ribosomes themselves was that, in a few instances, there was some increase in the relative concentration of ribosomal subunits, probably through removal of Mg^{++}.

The results on ribosomal concentration (Table X) in the control homogenates not treated with DOC are similar to those of the previous experiments (Table IX). In the expanding coleoptiles (15 and 35 mm) the concentration of ribosomes remained relatively constant, while in the fully grown coleoptiles (60 mm) a drop in ribosomes was evident. The effect of DOC on homogenates from the young coleoptiles was variable and relatively small, indicating that the bulk of the ribosomes in the growing cells were

TABLE X

Concentrations of free ribosomes in coleoptiles before and after treatment of fractions with 0.3% DOC

Conditions of analysis as in Table VIII.

Coleoptile		Material treated with DOC	Concentration (area-arbitrary units)		% Increase in conc. with DOC
Age (days)	Length (mm)		−DOC	+DOC	
3	15	Whole homogenate	61	87	43
3	15	Whole homogenate	46	56	22
3	15	Microsomal pellet	42	54	29
4	35	Whole homogenate	43	39	−9
4	35	Whole homogenate	38	34	−10
4	35	Microsomal pellet	31	48	55
6	60	Whole homogenate	10	36	260
6	60	Whole homogenate	12	28	130
7	60	Whole homogenate	10	23	130

either not bound to membranes *in situ* or were easily removed from membranes by homogenization without DOC. On the other hand, in the fully elongated coleoptiles DOC seemed to give a consistently significant rise in ribosomal concentration in the homogenates. However, the most important finding from these experiments, in spite of their limited accuracy, was that both without or with DOC treatment, there was no evidence of an increase in ribosomal concentration in expanding coleoptiles.

DISCUSSION

Taken overall, the results clearly indicate that very little RNA synthesis occurs during the grand period of cell expansion following cell division in oat coleoptiles. The lack of sensitivity of expansion growth to 5-FU the relatively low incorporation of RNA precursors into RNA of expanding cells and the lack of increase in ribosomes during expansion all support this conclusion. It therefore seems that in this tissue the bulk of the RNA in the expanding cells is formed prior to the main period of cell expansion and persists, or slowly declines, during expansion.

This conclusion is in agreement with several independent lines of evidence. It is well known that young, dividing, plant cells stain strongly for RNA with basic dyes such as Azure B at pH 4. However, this intense basophilia diminishes as cells expand so that fully expanded plant cells give only a weak stain in their thin cytoplasmic layer. Using the electron microscope numerous workers (*16–18*) have found that the cytoplasm of young meristematic plant cells is very rich in ribosomes, while in older vacuolated plant cells the ribosome population is much less obvious. In oat coleoptile cells viewed in the electron microscope the transition from densely packed ribosomes in young cells to infrequent, widely spaced ribosomes in vacuolated cell is clearly evident (*18*). These observations are consistent with the idea that during cell expansion ribosome synthesis either ceases or declines markedly and pre-existing ribosomes are diluted in an increasing cytoplasmic volume to give an overall lower ribosome concentration, per unit cytoplasmic volume.

WOODSTOCK and SKOOG (*19*) have come to a somewhat similar conclusion regarding the relationship between RNA synthesis and growth in corn roots. They found that the amount of growth of the roots of different corn varieties was directly correlated with the amount of RNA synthesis occurring in the apex of the root in the cellular stage preceding rapid expansion. They also reported on the basis of autoradiographic experiments with ^{3}H-cytidine that RNA synthesis was restricted to the root apex below the region of maximum elongation. HOLMES et al. (*20*) using direct phosphorous determinations in broad bean roots reported that RNA per cell reached a maximum in the early elongation stage and then showed a slow decline into the region of maturation. Their data, in fact, very closely parallel the ribosomal analyses presented here. JENSEN's data with onion roots (*21*) are also consistent with this pattern of RNA synthesis although his analysis did not

extend into the main elongation zone. SUNDERLAND and MCLEISH (*22*), on the other hand, analyzed the RNA contents of a number of different root meristems and found considerable variation in net RNA increases in relation to stage of growth. In broad bean, corn and pea roots the RNA per cell increased within 5 mm of the tip and then remained more or less constant in the region above this, while in other species net increases in RNA occurred through the entire elongation region. HEYES (*23*) found, in contrast to SUNDERLAND and MCLEISH, that a relatively large increase in RNA occurred throughout the elongation zone of pea roots. Some of the above conflictions in data probably result from different methods of determining RNA amounts but it does seem possible that differences in amount of RNA synthesis in different cellular stages can occur in different species. However, there does seem to be considerable evidence from root tip studies to indicate that in most species the majority of RNA synthesis occurs during cell division and early elongation stages and subsequent elongation proceeds with either a relatively constant or only slowly changing content of RNA.

Studies by KEY and associates on growing shoot systems also indicate that increases in total RNA need not accompany cell expansion. Intact corn mesocotyls show a decline in total RNA content per cell in the elongation region, and auxin treatment of excised mesocotyls accelerated the rate of RNA loss (*24*). Excised soybean hypocotyls also failed to show a net increase in RNA even when supplied with concentrations of auxins which promoted optimal cell expansion (*25*).

The above studies on levels of total cellular RNA, using relatively unspecific assay procedures for nucleic acid, probably concern largely ribosomal, and to a lesser extent transfer, RNA's. In general the studies indicate that synthesis of these types of RNA need not, and usually does not, take place during the grand period of plant cell expansion. The data presented here on ribosomal concentrations and isotope incorporation supports this conclusion directly for oat coleoptiles. Furthermore, the insensitivity of coleoptile growth to treatment with 5-FU is quite understandable in the light of the recent finding by KEY and INGLE (*6*) that in plant tissues 5-FU selectively disrupts ribosomal and transfer RNA synthesis without significantly influencing synthesis of possible messenger RNA. Since ribosomal synthesis has essentially ceased in expanding coleoptiles 5-FU would not be expected to have an inhibitory action on growth through disruption of ribosome synthesis. On the other hand, ribosome synthesis is probably essential for normal cell division in meristems, certainly autoradiographs indicate that extensive RNA synthesis occurs in dividing root tip cells (*11, 19*), and 5-FU might be expected to be inhibitory toward root growth, as found here. However, the fact that 5-FU had no inhibitory action toward intact coleoptiles on seedlings suggests that ribosome synthesis may not be important in the dividing cells of young coleoptiles. Indeed, it is possible that the coleoptile of the mature embryo has a complement of ribosomes sufficient to support all protein synthesis necessary for complete growth

of the organ. The fractionation analysis of ribosome concentration in 3 mm coleoptiles (Table IX), in which cell division is occurring, would support this hypothesis.

This situation, where ribosome synthesis precedes growth, is strikingly similar to that found in amphibian zygotes, which contain sufficient ribosomes to support embryonic growth to the blastula stage in the absence of ribosome synthesis (*26*). However, in mutants incapable of ribosome synthesis, ribosome content becomes a limiting factor in growth beyond the blastula stage. It is possible that ribosome content may also limit growth in plant cells. For example, differences in growth potential of the corn mutants studied by WOODSTOCK and SKOOG (*19*), which were correlated with RNA content of the meristems, might be determined by inherited differences in ribosome content of the cells at the start of expansion. Results on inhibition of expansion in Jerusalem artichoke tuber cells by 5-FU (*4*) are also interpretable on this basis. If 5-FU is supplied prior to cell expansion, during a period of rapid RNA synthesis, subsequent auxin-induced synthesis is severely inhibited, presumably due to prior disruption by 5-FU of ribosome synthesis. Similar inhibition of expansion by pretreatment with RNase (*2*) might also reflect ribosomal limitation of growth.

Apart from these considerations of ribosomal metabolism in growing cells there is ample evidence that some RNA synthesis during cell expansion is essential for expansion to proceed. The autoradiographic evidence presented here consistently indicated a small incorporation of labelled precursors into RNA of nuclei of expanding cells. The fact that significant incorporation was not usually found in cytoplasm also does not necessarily indicate that the RNA being synthesized in the nuclei remained there. If the small amount of nuclear incorporation was continually dispersed in the cytoplasm, where it might also turn over, it would not be detectable by autoradiography, particularly in the presence of the relatively high background due to the incorporation of isotope into non-RNA fractions. In the actinomycin D experiment where ^{3}H-cytidine was supplied for 24 instead of 5 hr, measurable labelling of cytoplasmic RNA occurred. Most significantly however, actinomycin D markedly reduced the nuclear and cytoplasmic RNA labelling and at the same time inhibited cell expansion. Inhibition of cell expansion by actinomycin D has also been reported by NOODÉN and THIMANN (*7*), KEY (*5*) and CLELAND (*8*). KEY and INGLE (*6*) have analyzed the effect of actinomycin D on various RNA fractions in expanding plant cells and conclude that this antibiotic, unlike 5-FU, inhibits synthesis of a DNA-like RNA which is essential for cell expansion. The actinomycin D sensitive incorporation of RNA precursors detected in the present study undoubtedly represents synthesis of this RNA fraction; presumably a messenger type RNA. The relatively low absolute incorporation of isotope and the absence of preferential nucleolar labelling are quite consistent with this interpretation.

Apparently then, as KEY and INGLE (*6*) conclude, continued production

of messenger RNA is necessary for expansion of plant cells to proceed normally. This RNA presumably couples with pre-existing ribosomes to mediate synthesis of proteins necessary for cell development. NOODÉN and THIMANN (*7, 27*) and KEY (*5*) have presented convincing evidence that continued protein, as well as RNA, synthesis is necessary for cell expansion. The present studies indicate that protein synthesis might even accelerate as coleoptiles grow. In addition, production of messenger RNA and protein apparently does not necessarily stop with cessation of cell expansion. The fully expanded coleoptile cells studied here continued to synthesize RNA and proteins. KEY and SHANNON (*25*) also found continued RNA synthesis in fully expanded soybean cells. Presumably the RNA produced after expansion has ceased represents products of genes responsible for further cell differentiation and is qualitatively distinct from the RNA produced during expansion. This, however, remains to be demonstrated.

The relationship of auxin to RNA remains obscure. RNA synthesis in soybean hypocotyls is definitely stimulated by auxin (*25*) and KEY (*5*) has suggested that regulation of RNA synthesis may be a basic action of auxin. A similar suggestion has also been made by ROYCHOUDHURRY et al. (*28*) and DATTA and SEN (*29*) on the basis of their finding that IAA stimulates both RNA and protein synthesis in coconut milk nuclei. Stimulation of both nuclear RNA and DNA synthesis has been cytochemically demonstrated in Jerusalem artichoke tissue but there the nucleic acid responses seem to be associated with auxin stimulation of cell division rather than cell expansion (*4, 30*). The fact is that the autoradiographic procedures which readily reveal auxin stimulation of nuclear RNA synthesis in dividing artichoke cells (*4*) failed, in the present study, to give clear-cut evidence of an auxin effect on RNA synthesis in the expanding cells of oat coleoptiles. Similarly, biochemical analyses failed to reveal promotion of RNA synthesis by auxin in corn mesocotyls (*24*). Thus, although mediation of RNA synthesis would seem to be an attractive hypothesis for a metabolically central action of auxin, the available evidence, which is quite limited, is equivocal on this point. It seems entirely possible that actual stimulation of RNA synthesis by auxin, as distinct from the RNA synthesis necessary for auxin action, may be a secondary effect of auxin observable only in some cells, just as is DNA synthesis (*4*). At the moment it seems more attractive to suppose that auxin, if it has a single central role in growth metabolism, interacts with pre-existing RNA's and in some way influences protein synthesis such as to bring about cell development. Presence of different RNA's in different cells could then lead to qualitatively different cellular responses to auxin, as found in Jerusalem artichoke cells (*4*). Such a general hypothesis would appear to be at least consistent with the results of a number of workers (*1–5, 7, 8, 27, 30, 31*) and with those of the present study.

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