M.Sc. ABSTRACT Biology

RIBONUCLEIC ACID AND RIBONUCLEASE LEVELS IN THE PEA EPICOTYL FOLLOWING TREATMENT WITH VARIOUS GROWTH REGULATORS

Growth responses and levels of RNA and RNase activity were measured in subcellular fractions of the growing region of decapitated Pisum sativum epicotyls following treatment with various growth regulators. Auxin-treated tissue swelled and developed higher total RNase activity than controls. There were comparable increases in RNA and protein. A marked unilateral rise in RNase activity (e.g., 20-fold) was associated with a "heavy" ribosomal membrane subfraction of the microsomes.

Other growth regulators had no such effects. Treatment with benzyladenine (BA) prevented the losses of RNA and protein which occurred in controls, but total and microsomal RNase activity were not affected. When BA was added together with auxin, effects of the two growth regulators on growth and RNA and protein increments were more than additive, and the great accumulation of RNase activity in the microsomal fraction which usually followed auxin treatment was completely abolished. Polysomal profiles prepared after auxin plus BA treatment showed less degradation than those from any other treatment. It is concluded that treatment with auxin generates and BA suppresses the RNase activity found in a membrane-bound fraction. This activity may have a function in control of the "turnover" of auxin-evoked polysomes required for growth.

M.Sc. Biology

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Suggested Short Title:

"Ribonuclease in the Pea Epicotyl"

Ribonucleic Acid and Ribonuclease Levels in the Pea Epicotyl Following Treatment with Various Growth Regulators

by

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PREFACE

In keeping with the newly-accepted regulations for thesis style which have been authorized by the Graduate Training Committee of the Biology Department at McGill, the main body of this thesis has been written in a form suitable for publication. Use is made of the style recommended by the journal Plant Physiology and, with minor changes, it is due to be submitted to that journal for publication. At relevant points in the text, reference is made to appendices where further details of the points under consideration are given. This includes a more extensive citation of literature and supplementary experimental data from results obtained in this study and not reported elsewhere.

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Generation and Suppression of Microsomal Ribonuclease Activity in the Decapitated Pea Epicotyl Following Treatments with Auxin and Cytokinin[1]

by

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ABSTRACT

Growth responses and levels of RNA and RNase activity were measured in subcellular fractions of the growing region of decapitated Pisum sativum epicotyls following treatment with various growth regulators. Auxin-treated tissue swelled and developed higher total RNase activity than controls. There were comparable increases in RNA and protein. A marked unilateral rise in RNase activity (e.g., 20-fold) was associated with a "heavy" ribosomal membrane sub-fraction of the microsomes.

Other growth regulators had no such effects. Treatment with benzyladenine (BA) prevented the losses of RNA and protein which occurred in controls, but total and microsomal RNase activity were not affected. When BA was added together with auxin, effects of the two growth regulators on growth and RNA and protein increments were more than additive, and the great accumulation of RNase activity in the microsomal fraction which usually followed auxin treatment was completely abolished. Polysomal profiles prepared after auxin plus BA treatment showed less degradation than those from any other treatment. It is concluded that treatment with auxin generates and BA suppresses the RNase activity found in a membrane-bound fraction. This activity may have a function in control of the "turnover" of auxin-evoked polysomes required for growth.

There is a growing literature confirming that long-term control of growth by hormones is exercised through actions on nucleic acid metabolism in both plant and animal systems (15,34,37). Plant hormones often evoke RNA synthesis, both messenger and ribosomal, and new polysomal species presumably code for enzymes that help to bring about growth (10). It should also be recognized that precise regulation of enzyme synthesis can result from control of stability of messenger RNA (36). At least in animal tissues, such control can be effected by hormonal regulation of RNase levels at crucial times and locations in growing cells (4). In different plant tissues, however, effects of various hormones on RNase activity appear to be contradictory, (15,37) and a precise role for this enzyme during growth has not been established for any one system.

For example, concurrent increases in RNA, protein and RNase activity in response to auxin treatment have been reported for corn mesocotyl (29) and barley and wheat leaves (21). In bean leaves, however, Fletcher (1969) clearly demonstrates that these responses follow cytokinin treatment, which suppresses total RNase activity in barley and wheat (30,31,32). In further contrast, Key and Shannon (1964) report large increases in RNA and protein levels in auxin-treated soybean hypocotyl without concurrent increases in RNase activity. Inhibition of RNase activity after auxin treatment is reported for bean endocarp (28), bean petioles (1) and soybean seedlings (21). Undoubtedly, whether auxins or cytokinins stimulate or inhibit RNA metabolism depends in part on their relative concentrations (26,39).

Another source of confusion may be the fact that, in the majority of developmental studies, only total RNase activity has been measured, and in cases where tissue is fractionated, often only one fraction is investigated. Whenever plant RNase has been analysed with any degree of sophistication, more than one RNase has been demonstrated to co-exist in the same tissue (24,27,40,42) and, in most cases, the various RNases have been associated with either the soluble or membrane-bound fractions (e.g., microsomes).

To date, a thorough investigation of the effects of different growth regulators on total and subcellular levels of RNA and RNase activity, and their relation to growth in a well characterized tissue has not been reported. This paper describes effects mainly of auxin and cytokinin, alone or together, on these parameters in growing regions of decapitated pea epicotyls. In this semi-intact system, auxin treatment has been shown (6,9) to result in lateral cell expansion and massive increases in RNA and protein in various cell subfractions, including microsomes.

MATERIALS AND METHODS

Growth and Treatment of Peas. Seedlings of Pisum sativum L., var. Alaska were grown in darkness for 8 days. Under dim green light, those with third internodes 3 to 5 cm long were decapitated just below the hook. A point on the epicotyl 10 mm below the apex was marked with ink to delineate a "segment" of tissue. Each apex was painted with about 2.5 mg lanolin paste (70% w/w water) ± approximately 10 µg growth regulator, and

seedlings were allowed to continue growth in darkness at 22 C. Three days later, at least 50 segments per treatment were detached, lanolin was wiped from the surface and length and fresh weight were recorded.

Isolation of "Light" and "Heavy" microsomes. Segments (approximately 5 g fresh wt/treatment) were ground in a mortar at 4 C in approximately 2 volumes of extraction medium consisting of 0.4 M sucrose (ribonuclease-free), 5 mM Mg acetate, 100 mM Tris-HCl (pH 7.5 at 22 C), 20 mM KCl, and 5 mM β-mercaptoethanol. The brei was squeezed through Miracloth (Calbiochem) to remove cell walls and debris (wall fraction) and the exudate was centrifuged at 16,000g for 10 min to sediment mitochondria and nuclei (particulate fraction). The resulting supernatant was divided into two 6-ml aliquots and layered over 6 ml 50% (w/v) sucrose (ribonuclease-free) dissolved in resuspension medium [10 mM Tris-HCl (pH 7.5 at 22 C), 10 mM KCl, 2.5 mM Mg acetate and 1 mM β-mercaptoethanol]. The tubes were centrifuged in an IEC SB rotor No. 283 at 40,000 rpm (190,000g_{av}) for 3 hr. A band of material approximately 2 mm thick was visibly held back by the 50% (w/v) sucrose interface. This was collected readily with a Pasteur pipette and is referred to here as the "light" microsomal fraction (contaminated with supernatant). The pellet sedimenting at the bottom of the tubes is termed the "heavy" microsomal fraction.

To prepare "washed heavy" microsomes, pellets were dispersed in resuspension medium by agitation for 2 sec in a Polytron PT 10/ST Homogenizer. A 6-ml aliquot was layered

over 6 ml 20% (w/v) sucrose dissolved in resuspension medium and recentrifuged as above for 1 hr. For protein, nucleic acid and RNase assays, both "light" and "heavy" microsomes were resuspended and homogenized using the Polytron.

Preparation of Polysomal Profiles. The methods used were modified from those described by Wettstein et al. (1963). Aliquots (6 ml) of the 16,000g supernatant were layered over 6 ml 50% (w/v) sucrose dissolved in resuspension medium and centrifuged in an IEC angle rotor No. 169 at 40,000 rpm (145,000g_{av}) for 1 hr. The resulting pellet was gently resuspended in 0.5 ml resuspension medium with a round-tipped glass rod and was layered over a 12-ml linear gradient of sucrose (10 - 35%, w/v) in resuspension medium underlaid by 0.5 ml 56% (w/v) sucrose. This was centrifuged in an IEC SB rotor No. 283 at 30,000 rpm (105,000g_{av}) for 2.5 hr. at 0 C. The gradient tubes were punctured and the absorbance of the effluent was scanned continuously at 254 nm using an ISCO model D density gradient fractionator. The effluent was monitored at a speed of 2 ml/min and recorded at a chart speed of 1 inch/min.

Protein and Nucleic acid estimation (cf. Appendices I to IV). Aliquots of subcellular fractions were precipitated with equal volumes of ice-cold 10% (w/v) trichloroacetic acid (TCA) and washed consecutively with cold 5% (w/v) TCA, absolute acetone and ether. Nucleic acid was extracted from the de-fatted precipitate in warm (70 C) 0.5N perchloric acid and estimated

by measuring OD_{260} minus OD_{290} values using wheat germ RNA (Calbiochem) as standard (14). Protein in the perchloric acid-insoluble residue was dissolved in 1N NaOH and estimated by the Biuret method (12) or the Lowry method (18) using bovine serum albumin as standard.

RNase activity. RNase activity was assayed using a modification of the methods of Zittle (1946) and Lyndon (1966). The reaction medium consisted of 0.2 ml suspended subcellular fraction, 0.5 ml 0.2 M Tris-acetate, pH 6.0, and 0.1 ml 2% (w/v) wheat germ or yeast RNA (highly-polymerized Calbiochem). The mixture was incubated at 35 C for 35 min and the reaction was stopped by adding 1.0 ml ice-cold 0.375% (w/v) uranyl acetate dissolved in 10% (w/v) TCA and 0.1 ml conc. HCl. Reaction mixtures were left to precipitate at 4 C for 30 min and centrifuged at 37,000g for 5 min. The supernatant was diluted with 10 volumes of distilled water and OD_{260} minus OD_{290} was measured using a Beckman DB-G spectrophotometer. One µg RNA hydrolysed to acid-soluble products under the above conditions was equivalent to an increment in the value for OD_{260} minus OD_{290} of 0.014 units, and was defined as one unit of RNase activity. RNase activity in the most active pea fractions was proportional to the time of incubation and concentration of the enzyme (cf. Appendix V). The pH (6.0) used in this assay was a compromise between the pH optima reported earlier for soluble pea RNase (pH 5.6), (13,24) and microsomal pea RNase (pH 5.9 - 6.2), (19,24).

RESULTS

Survey of Effects of Growth Regulators in the Decapitated Epicotyl (cf. Appendices VI and VII).

When plumule and hook were excised from 8-day old etiolated pea seedlings, and the cut apex was painted with lanolin paste containing no growth regulators (control), tissues in the apical 10 mm of the epicotyl showed changes often associated with senescence. In the 3-day period following treatment, these changes included (Table I): little further growth by elongation, swelling or cell division (DNA was constant); marked decreases in total protein and RNA; and an increase (doubling) in total RNase activity. The latter was the result of a gradual drifting upwards of RNase levels (Table II) which did not resemble the sigmoidal increment typical of wound responses (2,8).

The addition of GA and BA to the cut apex resulted in pronounced elongation and swelling respectively, (Table I). Both regulators had "protective" effects on protein and RNA levels, yet neither altered the total RNase levels (Table I).

The addition of IAA to decapitated epicotyls, however, caused swelling, massive rises in protein, RNA and DNA levels (9) and a substantial enhancement of RNase levels over control values (Table I). The latter increase was gradual and continued for at least 4 days (Table II). The herbicide 2,4-D, evoked similar responses (Table II). The increment in RNase activity was particularly striking in the microsomal fraction of the

tissue.

The addition of both IAA plus BA to the decapitated apex resulted in the greatest swelling and increments in protein, RNA and DNA levels that were observed throughout this study. The effects of the two growth regulators were more than additive (Table I). The total RNase activity, however, did not reach as high a level as it did in the presence of IAA alone. BA completely suppressed the IAA-evoked rise in RNase activity in the microsomal fraction.

Properties of Subcellular Fractions.

Figure 1 shows protein and nucleic acid levels recovered in fractions extracted from epicotyl segments after treatment with IAA and/or BA. Generally these components in each fraction decreased in controls, remained approximately constant upon treatment with BA alone, and increased after IAA treatment. The increases were especially pronounced after treatment with IAA plus BA. No attempt was made to subdivide the fraction containing cell wall material and large particulate debris (0 - 16,000g pellet). This fraction contained a large part (up to half) of the total nucleic acid and most (98%) of the tissue's DNA (6).

Microsomes were separated into "light" and "heavy" fractions which displayed RNA/protein ratios of 0.1 to 0.2, and 0.5 to 1.0 respectively. This indicates a relatively high content of membranes in the "light" fraction, compared with the "heavy" fraction (3,33). The increases in total RNase

levels which occurred in controls and in IAA-treated tissues (Tables I and II) were distributed throughout the above fractions (Table III). IAA effects were particularly marked in both "light" and "heavy" microsomes. The increase which occurred in BA-treated tissues was accounted for by increments in all fractions except the "heavy" microsomes. BA reduced the IAA-evoked RNase activity by an amount that could be accounted for by suppression of RNase activity in the "heavy" microsomal fraction. BA also caused a partial relocation of microsomal RNase activity into the soluble fraction.

Effects of "washing" microsome fractions on retention of RNase activity are shown in Table IV. Generally, RNase activity was easily removed from microsomal fractions simply by re-centrifugation through 20% (w/v) sucrose, but in microsomes extracted from IAA-treated tissue over half of the total activity remained associated with the pellet. Since "washing" also removed about half the total RNA and protein, the specific activity of RNase did not change in these microsomes. It is concluded that there exists a membrane-bound fraction of the total RNase activity which is evoked by IAA, suppressed by BA, and located in the microsomes.

Sucrose Density Gradient Profiles (cf. Appendix VIII).

Figure 2 shows the ribosome distribution of "heavy" microsomal fractions isolated from IAA and/or BA-treated epicotyl segments. A monosome peak occurs near the top of each gradient with a series of polysomal associations extending

towards the bottom. Yellow, opaque material sedimenting through the 56% sucrose "cushion" at the bottom of the tube was only partially recovered by the gradient fractionator. This material yielded a small peak of UV absorption and probably contained membrane-bound polysomes, comparable with the "heavy rough endoplasmic reticulum" of Tata and Widnell (1966).

Pellets isolated from epicotyl segments at zero day contained 80 to 85% of ribosomes as polysomes. In pellets isolated after 3 days from untreated segments, the profile indicated only 40% polysomes. Whereas BA treatment did not prevent this decline, IAA treatment partially reversed it (resulting in pellets with approximately 60% polysomes). Treatment with both IAA and BA preserved the polysome proportion present at zero day. Since IAA and IAA plus BA treatments greatly increased the RNA content of the "heavy" microsomes per segment (Fig. 1) the absolute yield of polysomes per segment increased 2 to 3 fold following such treatments. In repeats of this experiment, there were minor variations in values for percentages of polysomes, but relative effects of growth regulators were remarkably reproducible.

The location of RNase activity in relation to the polysomal profile was determined in a gradient prepared from "heavy" microsomes isolated from seedlings sprayed with 2,4-D. The profile (Fig.3) was similar to that found for segments treated with IAA plus BA, i.e., 77% polysomes. Approximately 40% of the RNase activity was located in the top 2 ml of the

centrifuge tube and represented soluble enzyme not sedimenting into the gradient. Another 40% precipitated with the pellet at the bottom of the tube. The remaining activity was spread diffusely throughout the gradient and showed no reproducible association with any peak.

DISCUSSION AND CONCLUSIONS

The RNase activity which is associated with microsomal fractions of the pea epicotyl is evidently controlled by two classes of plant growth regulators. Treatment with auxin (IAA or 2,4-D) greatly increases the total and specific RNase activities in microsomes, whereas treatment with equal amounts of cytokinin, alone or in combination with IAA, effectively suppresses this RNase fraction. These changes occur against a background of relatively high soluble RNase activity which increases gradually with age, particularly after treatments which cause protein levels to rise (Fig. 1, Tables I and III). No growth regulator caused an increase in the specific activity of soluble RNase activity in these tests. These observations are in keeping with the conclusion that there is more than one RNase in peas (19,24), and indicate that a microsomal RNase exists which is susceptible to antagonistic regulation by auxin and cytokinin.

Whatever the mechanisms may be whereby auxin and cytokinin control microsomal RNase activity, the consequences could be crucial for growth if a very high or very low RNase level were specifically associated with a fraction containing polysomes. There is no doubt that a primary action of auxin in

the pea epicotyl is promotion of RNA synthesis (6,9,23,25,38), specifically polysomes (6,38). Treatment with BA alone does not lead to polysome formation, but treatment with IAA plus BA results in increases in both the amount and proportion of auxin-evoked polysomes which can be recovered from the tissue (Figs. 1 and 2). Thus, it appears that auxin and cytokinin control polysome production and preservation respectively, and thereby both hormones may have a decisive influence on the rate of polysome turnover and growth.

To be sure, it can be calculated that there is sufficient potential RNase activity in microsomal fractions after auxin treatment to degrade all of the RNA in these fractions within one or two minutes (Fig. 1 and Tables I and III). This obviously does not happen. Auxin greatly enhances RNA levels in microsomal and other fractions (Fig. 1), and polysomal profiles were only partially degraded (Figs. 2 and 3). Moreover, the results of inhibitor studies (9,25) suggest that some of this new RNA is necessary for growth. It is necessary to suppose that auxin-evoked RNase activity is kept apart from RNA at least for a time, by compartmentalization. Certainly, much of the pea microsomal RNase activity is associated with a heavy membrane-rich subfraction (Fig. 3), and there is evidence (22) that soluble RNase is normally present in vacuoles. Accordingly, factors, including hormones, which control enzyme localization, e.g., by regulation of membrane properties, have the potential for modifying the results of hormonal controls over polysome longevity.

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Table I. Effects of Various Growth Regulators on Total Levels of Protein, Nucleic Acid and RNase Activity.

An apical segment (1 cm) was delineated on each decapitated epicotyl and the cut apex was painted with lanolin paste ± additives. At zero day (8 days old) and 3 days after treatment, segments were removed for measurements of length and fresh weight. Homogenates were prepared and total protein, RNA, DNA and RNase activity were assayed as described in "Materials and Methods". Microsomal fractions were obtained as a pellet after centrifugation of 16,000g supernatants through 50% (w/v) sucrose at 150,000g (i.e. = "heavy" microsomes). When seedlings were left to elongate undisturbed (intact), protein, nucleic acid and RNase levels in apical regions were similar to those recorded for GA-treated decapitated segments (cf. Appendix VIA). Fresh Wt/Length is a measure of the swelling response.

Treatment	Length	$\frac{\text{Fresh Wt}}{\text{Length}}$	Protein	RNA	DNA	RNase activity	
						Total	Microsomal
	mm	mg/mm		µg/seg		µg RNA hydrolysed/hr/seg	
Zero day	10.0	1.9	179	43.7	7.3	607	2
3 days							
Control	12.4	2.7	119	22.0	6.6	1142	19
+ Gibberellic Acid (GA)	19.8	2.8	263	32.0	7.0	1157	9
+ 2,4-D	12.5	3.2	291	82.3	13.7	1595	265
+ Indoleacetic acid (IAA)	12.5	4.9	343	100.2	13.8	2093	383
+ N^6-Benzyladenine (BA)	11.9	3.5	182	30.2	6.8	1211	9
+ IAA + BA	12.1	7.1	495	131.2	21.8	1708	11

Table II. Effect of Auxins on Total RNase Activity Levels in Decapitated Epicotyls.

This test was conducted with Alaska peas grown from younger pea seed than was used for other studies reported here; RNase levels were relatively high, probably due to natural variation between batches (17).

Time	RNase Activity		
	Control	+IAA	+2,4-D
days	µg RNA hydrolysed/hr/seg		
0	1930		
1	2860	3150	4150
2	2830	4130	6520
3	3660	5950	7340
4	3840	6575	----

Table III. RNase Activity of Subcellular Fractions of Apical Segments of Decapitated Epicotyls Following Treatment with IAA and/or BA.

Protein and nucleic acid levels in the fractions are shown in Fig. 1.

Treatment	RNase Activity				
	Walls + Particulate	Microsomes "Light"	Microsomes "Heavy"	Supernatant	Total
	µg RNA hydrolysed/hr/seg				
Zero day	226	5	2	511	744
3 days,					
Control	352	21	19	665	1057
+ IAA	728	238	386	962	2314
+ BA	330	62	6	736	1134
+ IAA + BA	797	94	17	1178	2086

Table IV. RNase Activity of "Washed" Microsomes.

The "heavy" microsomal fractions described in Table II and Fig. 1 were "washed" by centrifugation through 20% sucrose at 150,000g.

Treatment for 3 days	RNase Activity /seg	/µg protein	/µg RNA
	µg RNA hydrolysed/hr		
Control	2	1	1
+ IAA	258	20	26
+ BA	2	2	1
+ IAA + BA	4	1	1

Fig. 1. Protein and Nucleic acid levels in subcellular fractions of epicotyl segments following treatment with IAA and/or BA. Homogenates were filtered to remove walls and centrifuged at 16,000g (pellet = particulate fraction) and again at 190,000g through 50% sucrose (interface material = "light" microsomes, pellet = "heavy" microsomes); supernatant = 16,000g supernatant minus microsomes. Nucleic acid was estimated by O.D. measurement of warm perchloric acid extracts of the fractions; protein was estimated in residues by the Lowry method. RNase activities of these fractions are recorded in Table III.

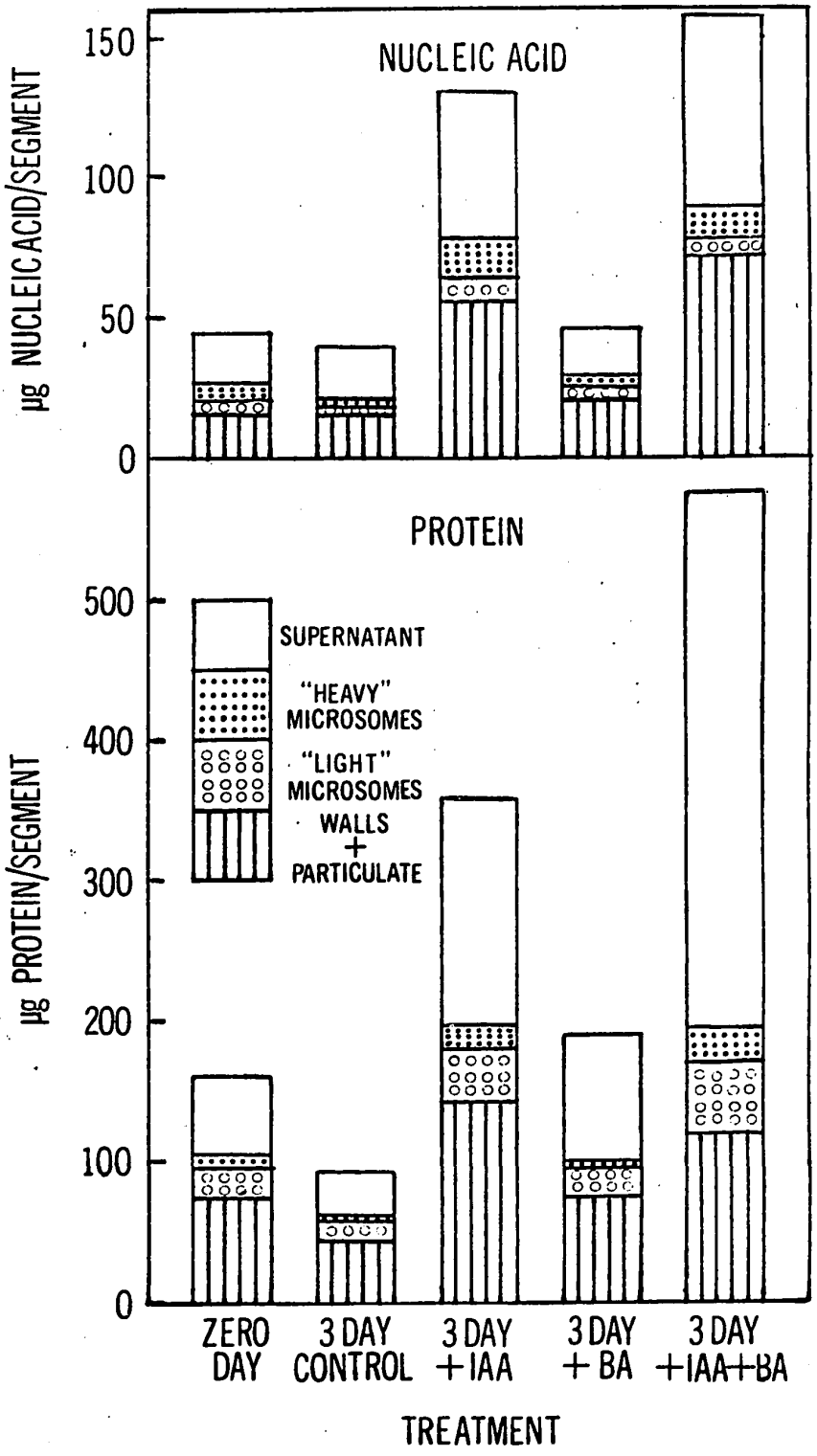

NUCLEIC ACID
µg NUCLEIC ACID/SEGMENT
150
100
50
0
PROTEIN
µg PROTEIN/SEGMENT
500
400
300
200
100
0
SUPERNATANT
"HEAVY" MICROSOMES
"LIGHT" MICROSOMES
WALLS + PARTICULATE
ZERO DAY
3 DAY CONTROL
3 DAY +IAA
3 DAY + BA
3 DAY +IAA+BA
TREATMENT

Fig. 2. Sucrose density gradient profiles of RNA derived from "heavy" microsomal pellets. Pellets from approximately 2g fresh wt were centrifuged through a linear sucrose gradient (10 - 35%) as described in "Materials and Methods". Direction of centrifugation is from left to right. The percentage of ribosomes occurring as polysomes is as follows: Zero day, 84%; 3-day control, 40%; +IAA, 61%; +BA, 34%; and +IAA+BA, 78%.

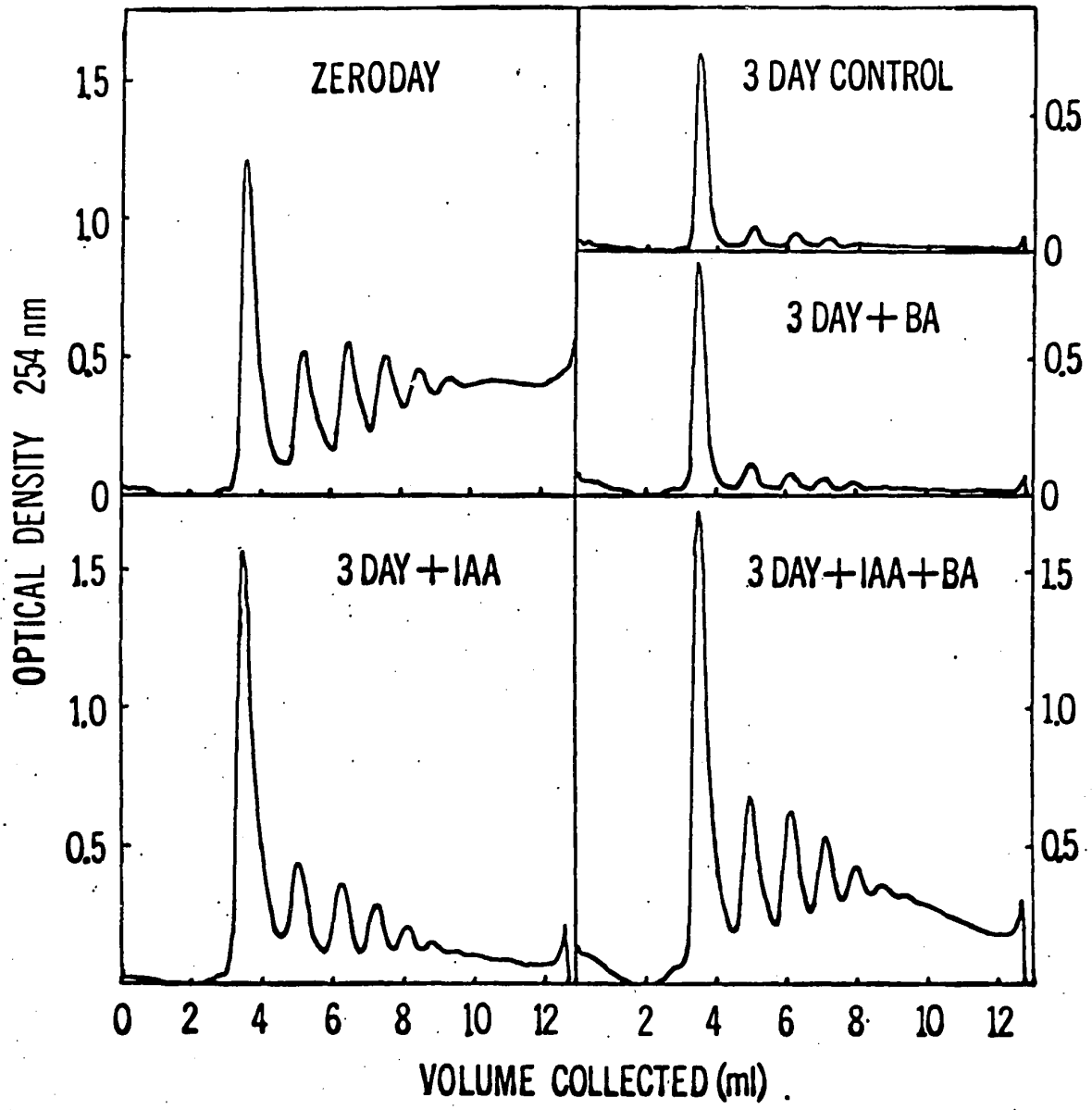
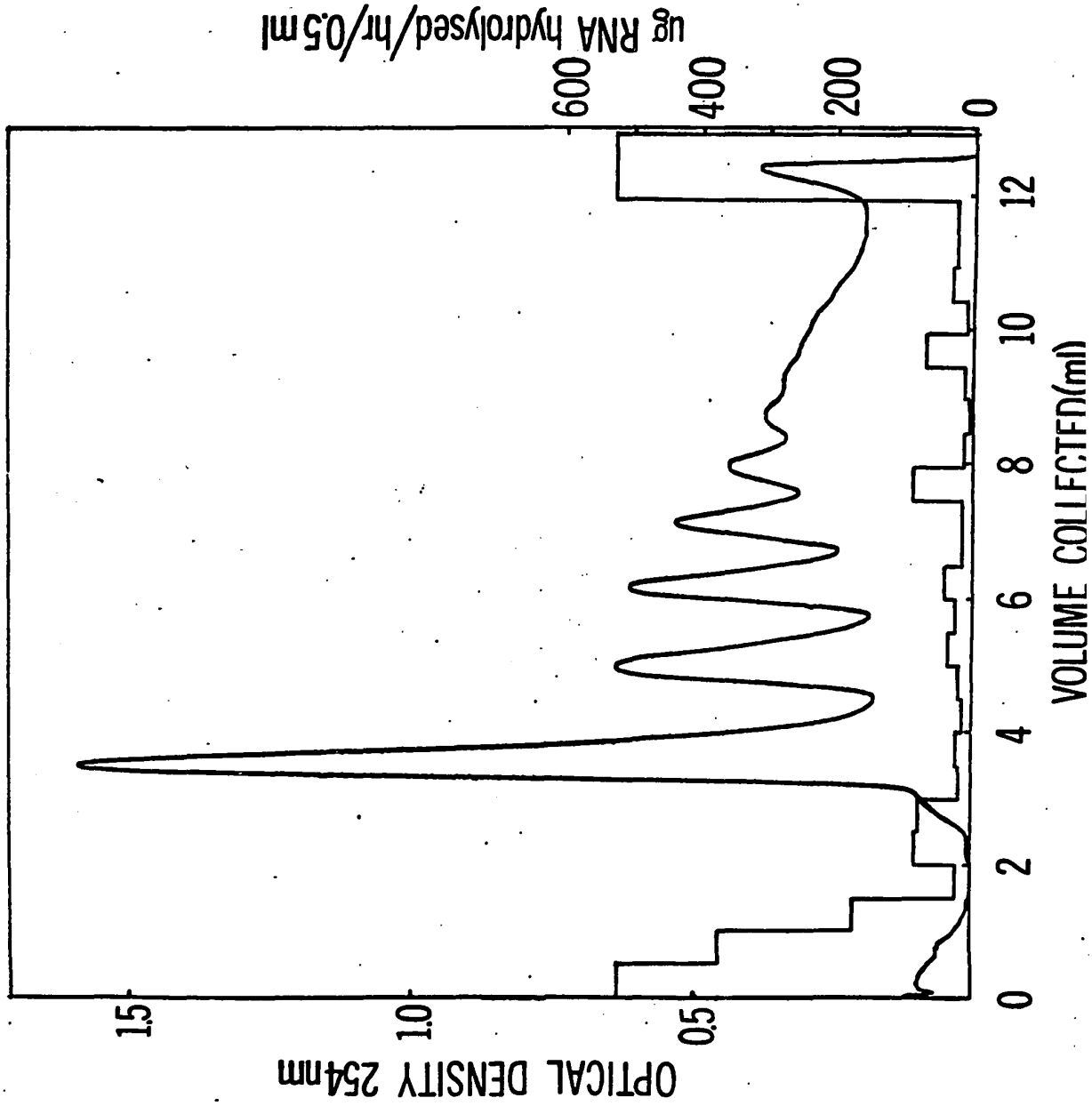

ZERODAY
3 DAY CONTROL
3 DAY + BA
3 DAY + IAA
3 DAY + IAA + BA
OPTICAL DENSITY 254 nm
VOLUME COLLECTED (ml)
0
0.5
1.0
1.5
2
4
6
8
10
12

Fig. 3. RNase activity distribution in a sucrose density gradient profile of "heavy" microsomes isolated from intact pea epicotyls following treatment with 2,4-D. 8-day old epicotyls were sprayed once with 0.1% (w/v) 2,4-D in 0.1 M NaCl. After 3 days, swollen regions of the epicotyl (subapical 10-12 mm) were excised, extracted and fractionated as described in "Materials and Methods". RNase activity was assayed in 0.5 ml aliquots of the gradient and is represented by the histograph.


µg RNA hydrolysed/hr/0.5 ml
600
400
200
0
OPTICAL DENSITY 254nm
1.5
1.0
0.5
0
2
4
6
8
10
12
VOLUME COLLECTED(ml)

Appendix I. Relationship between Protein Concentration and Optical Density as Measured by the Biuret Method.

For assay, 1.0 ml stock solution containing bovine serum albumin was precipitated with 0.2 ml cold trichloroacetic acid and centrifuged at 3,000g for 10 min. The supernatant was discarded and the precipitate resuspended in 3.0 ml water plus 3.0 ml Biuret reagent (9.0 g NaK tartrate, 3.0 g $CuSO_4.5H_2O$, 5.0 g KI/1 litre 0.2 N NaOH) and let stand at room temp. for 30 min, when $O.D._{545}$ was determined. Values are corrected for reagent blanks (6).

Protein (mg/ml)	$O.D._{545}$	$O.D._{545}$ per mg protein
1.0	0.041	0.041
2.0	0.080	0.040
3.0	0.115	0.038
4.0	0.158	0.040
5.0	0.196	0.039
6.0	0.242	0.040
7.0	0.282	0.040
8.0	0.314	0.039

Average value = 0.040 O.D. units/mg protein

Appendix II. Relationship between Protein Concentration and Optical Density as Measured by the Lowry Method.

For assay, 0.4 ml stock solution containing bovine serum albumin was mixed with 3.6 ml Lowry's reagent "C" [50 ml 2% (w/v) Na_2C0_3 plus 1.0 ml 0.5% (w/v) $CuS0_4.5H_20$ in 1% (w/v) NaK tartrate]. After 10 min at room temp., 0.4 ml Lowry's reagent "E" (0.1 N Folin reagent, Fisher Scientific Co.) was added with rapid mixing. After exactly 30 min, O.D. of the samples was read at 750 nm. Values were corrected for reagent blanks (11).

Protein (µg/ml stock solution)	$O.D._{750}$	$O.D._{750}$ per mg protein
40	0.074	0.18
80	0.146	0.18
120	0.213	0.17
160	0.280	0.17
200	0.342	0.17
240	0.404	0.16
280	0.472	0.16

Average value = 0.17 O.D. units/mg protein

Appendix III. Relationship between RNA Concentration and Optical Density.

For assay, stock solution of wheat germ RNA hydrolysed in warm (70 C) 0.5 N perchloric acid was used directly for O.D. measurements in a Beckman DB-G spectrophotomer.

RNA (µg/ml stock solution)	$O.D._{260} - O.D._{290}$	$O.D._{260} - O.D._{290}$ per µg RNA
2.5	0.036	0.014
5.0	0.071	0.014
7.5	0.104	0.014
10.0	0.141	0.014
20.0	0.262	0.013
30.0	0.422	0.014
40.0	0.529	0.013
50.0	0.677	0.014
60.0	0.867	0.014

Average value = 0.014 O.D. units/µg RNA

Appendix IV. Relationship between DNA Concentration and Optical Density as Measured after Reaction with Diphenylamine.

For assay, reaction mixtures containing 1 vol of DNA hydrolysed in warm (70 C) 0.5 N perchloric acid plus 2 vols Diphenylamine reagent (1.5 g diphenylamine dissolved in 1.5 ml conc. H_2SO_4 plus 100 ml glacial acetic acid, plus 0.1 ml freshly made aqueous acetaldehyde (16 mg/ml water) added to each 20 ml reagent) were incubated at 30 C for 18 hr and $O.D._{600}$ minus $O.D._{540}$ measured with a Bausch and Lomb "Spectronic 20" spectrophotomer. Values were corrected for reagent blanks (4).

DNA (µg/ml stock solution)	$O.D._{600} - O.D._{540}$	$O.D._{600} - O.D._{540}$ per mg DNA
10	0.034	3.4
20	0.067	3.3
40	0.144	3.6
80	0.293	3.6
120	0.424	3.5
160	0.515	3.2

Appendix V. Properties of Pea RNase Activity *in Vitro*.

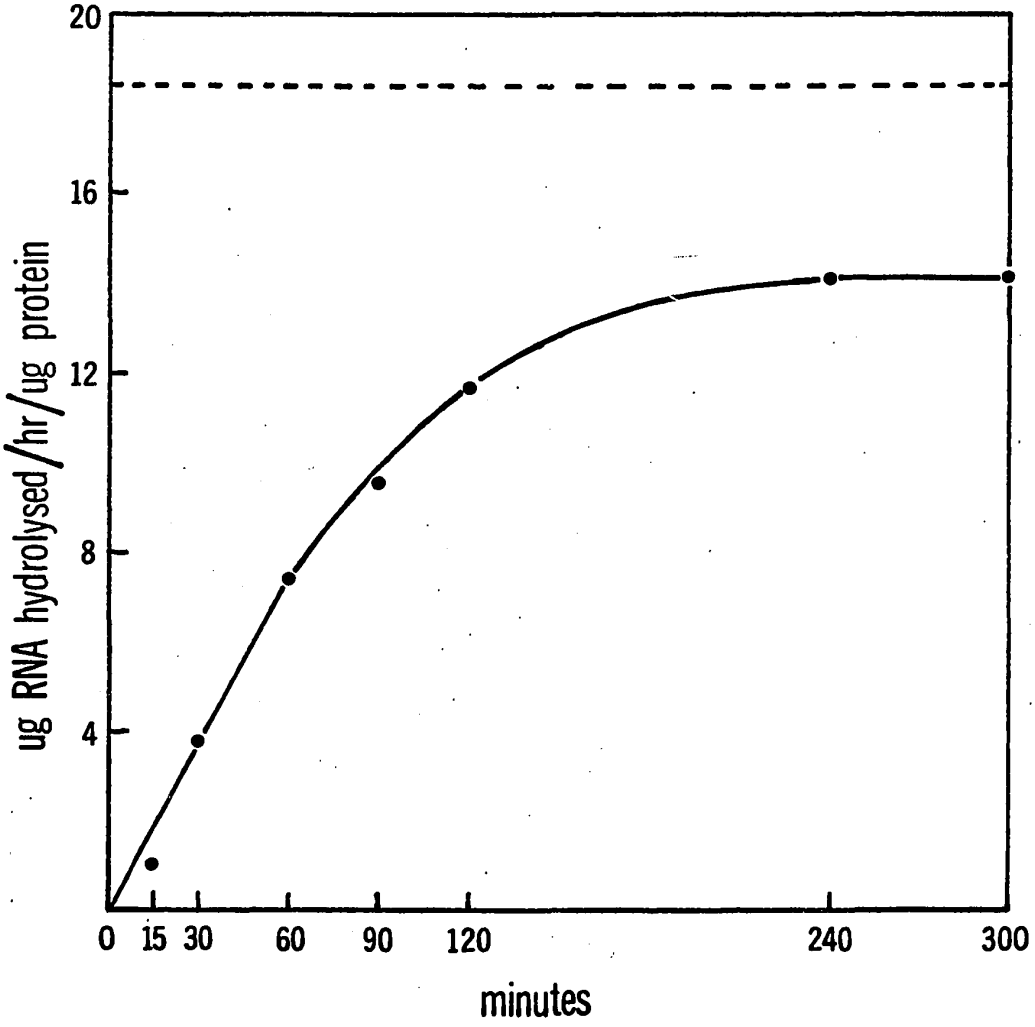
1. Progress of the RNase reaction.

Using the reaction mixture reported in the main body of the text plus supernatant enzyme extracted from 2,4-D-treated peas, progress of the reaction was followed for 5 hrs (Appendix VA). The rate of production of acid-soluble nucleotides was linear for the first hour and then levelled off during the next two hrs. The enzyme was unable to digest all the RNA substrate. No attempt was made to determine whether this was due to enzyme inactivation, end-product inhibition or resistance of an RNA "core" to RNase degradation.

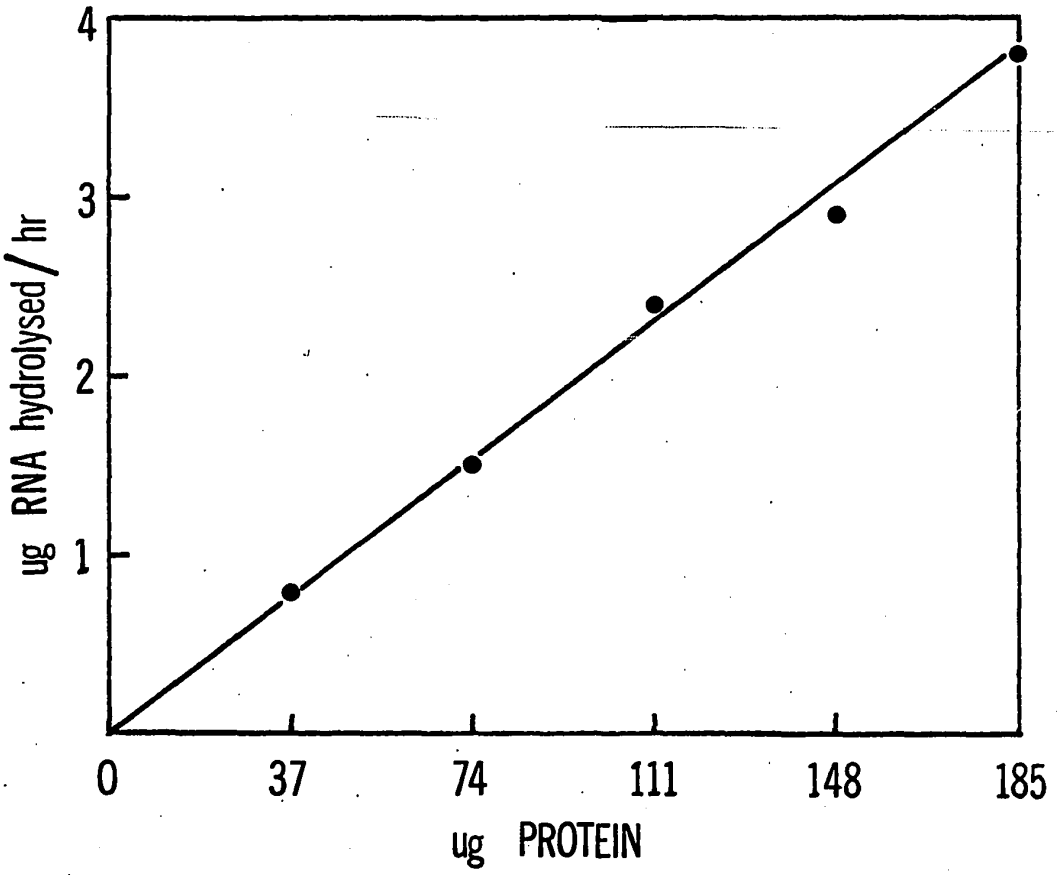
A reaction time of 35 min was arbitrarily chosen and used throughout these studies as a convenient assay period. The progress curve was linear for this period using the most highly active enzyme preparations that were encountered in the study.

2. Effect of Enzyme Concentration on Reaction Rate.

Appendix VB shows the results of an experiment using a serial dilution of the same enzyme preparation used in Appendix VA and the standard reaction procedure as reported above. Increasing the concentration of the enzyme caused a proportional increase in reaction rate up to at least 185 μg protein per assay tube. Protein levels in all assays reported in this thesis were less than this value.



Appendix VA. Progress of the RNase reaction.



Appendix VB. Effect of enzyme concentration on reaction rate.

Appendix VI. Effects of Various Growth Regulators on Growth and Total Levels of RNA, DNA, Protein and RNase Activity in the Decapitated Pea Epicotyl.

1. Survey of Growth Regulator Effects.

Appendix VIA records a summary of results obtained using Alaska peas received from our supplier (Asgrow Seed Co. - 90% germination) in 1969-70. In general, these peas gave very similar response patterns to added growth regulators as those reported in the main body of this thesis, which were grown from peas which were a year older. Using younger peas, growth responses were greater and levels of protein, RNA and RNase activity were all consistently higher. Ribosomal RNase levels have been shown (10) to vary widely between different batches of Alaska peas.

2. Effect of 2,4-D.

Appendix VIB records the results of a study of the effects of the auxin-herbicide 2,4-D on pea seedlings grown from the same batch of pea seed used in Appendix VIA. RNA, DNA, protein and RNase levels rose dramatically over the 3-day period but swelling and fresh weight increases were no greater than in controls (cf. IAA effects, Appendix VIA). 2,4-D-treated tissues turned brown and became flaccid in 3 days.

3. Effect of BA added together with IAA.

Appendix VIC records the results of an experiment investigating the effects of adding increasing concentrations of BA together with IAA to the decapitated epicotyl. Even at

concentrations 1/1000th that of IAA, BA had distinct effects on swelling, RNA, DNA and protein levels. Similar effects of cytokinin plus auxin on swelling have been recorded using excised pea epicotyl sections (19). When BA was added at equal concentrations to that of IAA, additive responses by these components were observed.

Appendix VIA. Effects of Various Growth Regulators on Growth and Total Levels of RNA, DNA, Protein and RNase Activity in Decapitated Pea Epicotyls.

Segments 10.5 mm long were marked at the decapitated apex of the third internode of 8-day old etiolated pea epicotyls, and growth regulators (10 µg/epicotyl) applied in lanolin paste. In the case of ethylene treatment, pea seedlings were grown in large glass jars and decapitated and painted when the second internode was 1 to 3 cm long. The jars were then covered with plexiglas, sealed with silicone grease and ethylene gas (10 ppm) injected with micro-syringes through vaccine caps fitted into the covers. The gas mixture was changed twice daily to reduce fungal infection and improve growth by flushing the jars with air and re-injecting the ethylene gas. After 3 days treatment, segments were removed for measurement of length and fresh weight, and homogenized in 1 to 2 vols of cold 0.4 M sucrose, 5 mM Tris-HCl (pH 7.4 at 22 C), 1.5 mM $MgCl_2$ and 0.02% (w/v) Na deoxycholate using a "Virtis 45" homogenizer run at top speed for 5 min. The homogenate was used directly for RNA, DNA, protein and RNase activity estimations as described above.

Appendix VIA. Effects of Various Growth Regulators on Growth and Total Levels of RNA, DNA, Protein and RNase Activity in the Decapitated Pea Epicotyl.

Treatment	Length	$\frac{\text{Fresh Wt}}{\text{Length}}$	Protein	RNA	DNA	RNase Activity
10µg/epi	mm/seg	mg/mm		µg/seg		µg RNA hydrolysed/hr/seg
Zero day	10.5	2.3	277	46	7.3	1660
3 days,						
Control (decapitated)	12.9	3.7	260	32	6.6	3100
Control (intact)	27.5	2.2	343	33	6.1	4675
+ E (10 p.p.m.)	15.5	4.0	344	42	7.1	3260
+ GA	26.3	3.8	357	41	6.3	5915
+ 2,4-D	12.0	4.4	681	110	14.0	7340
+ IAA	12.5	6.2	585	90	14.0	7050
+ BA	13.3	4.2	331	41	6.8	2940
+ IAA + BA	11.7	9.6	1053	155	22.0	6690

Appendix VIB. Effect of 2,4-D on Growth, RNA, DNA, Protein and RNase Activity in Decapitated Pea Epicotyls.

Segments 11 mm long were marked at the decapitated apex of the third internode of 8-day old etiolated pea epicotyls. They were painted with lanolin ± 2,4-D and treated segments were removed daily for measurements of length and fresh weight. The tissue was homogenized and extracted as described in Appendix VIA.

Treatment	Length	$\frac{\text{Fresh Wt}}{\text{Length}}$	Protein	RNA	DNA	RNase Activity
10µg/epi	mm/seg	mg/mm		µg/seg		µg RNA hydrolysed/hr/seg
Zero day	11.0	2.6	365	57	7	3198
1 day + 2,4-D	12.2	4.4	517	69	10	4152
2 days + 2,4-D	12.2	4.4	663	112	14	6525
3 days + 2,4-D	12.0	4.4	681	110	14	7339
3 days, control	15.2	3.7	333	36	7	3745

Appendix VIC. Effects of Increasing Amounts of BA Added Together with IAA.

Treatment	Length	$\frac{\text{Fresh Wt}}{\text{Length}}$	Protein	RNA	DNA
µg/epi	mm/seg	mg/mm	µg/seg		
Zero day	11.0	2.4	339	54	11
3 days					
Control	13.2	3.4	257	29	7
+ 10 µg BA	12.0	4.3	336	41	7
+ 10 µg IAA	13.0	5.3	718	109	16
+ 10 µg IAA + 0.01 µg BA	13.4	7.4	823	133	18
+ 10 µg IAA + 0.10 µg BA	12.2	8.6	1057	167	21
+ 10 µg IAA + 1.00 µg BA	12.4	9.9	1184	181	25
+ 10 µg IAA +10.00 µg BA	12.3	10.9	1259	190	25

Appendix VII. The development and Distribution of RNase Activity in the Pea Epicotyl.

1. Total RNase Activity Development during Pea Seedling Growth.

Following 17 to 24 hrs imbibition, germination is initiated in the pea seed (21). During the two days following germination, there is a rapid rise in the RNA and protein content of the cotyledons and a smaller rise of these components in the axis (1). About four days after germination, RNA and protein levels drop in the cotyledons, and there is a corresponding increase of these components in the axis (1).

RNase activity on a fresh weight basis rises rapidly in the cotyledons to a maximum about four days after germination, however the specific activity continues to rise (1,2,7). On a fresh weight basis, the RNase activity in the axis rises almost as rapidly as in the cotyledons, levelling off about seven days after germination, the activity then rises or falls depending on whether the seedling is grown in the dark or in the light. In the latter case, the RNase activity rises (7), and in the former, it falls (3). The specific activity of axis RNase in both light and dark grown peas, rises sharply until about the sixth day after germination, and then rises more gradually, (1,2,3).

RNase activity is low in the embryo and increases with the RNA and protein levels in the young and developing tissue. This suggests that RNase plays an initial role in the turnover of RNA in rapidly synthesising tissues and is probably

relocated in the vacuole as the tissue matures (cf. Appendix VIIB). The role of RNase in mobilising cotyledon reserves is more complex because RNase activity only rises after most of the RNA in the cotyledon has been depleted (1,3) and only a small percentage of labelled nucleotides incorporated into cotyledon RNA appears in the axis RNA (3).

2. The Distribution of RNase Activity in the Pea Epicotyl.

Several workers have reported gradients of RNase activity between meristematic and mature regions of the growing plant [corn root tip, (8,13); corn mesocotyl, (18); lentil root tip, (16)]. Maroti (1969) has attempted to correlate RNase activity with growth. Results of a similar investigation of meristematic, elongating and maturing regions of the etiolated pea epicotyl are shown in Appendix VIIA. In all of these tissues, when RNase activity is expressed on a fresh weight basis, there appears to be a decline in activity with age of the tissue. However the specific activity of the enzyme and its activity per cell (or per μg DNA) show steady increases during elongation, with a maximum at early maturity, and a levelling off in more mature cells. During this developmental process, major increases in the volume of the vacuole occur. This organelle is associated with high levels of RNase activity (14,15), which suggests a possible correlation between RNase activity and vacuolation rather than with growth per se.

Appendix VIIA. Fresh Weight, RNA, DNA, Protein and RNase Activity of Pea Epicotyl Sections.

8-day old etiolated pea epicotyls with third internodes 3-5 cm long were cut into sections with razor blades (12). Sections were weighed and then ground in a chilled mortar with 1 to 2 vols of extraction medium (cf. Appendix VIA). Total RNA, protein and RNase activity were estimated as described above.

Component	Plumule & Hook	0-1 cm	1-2 cm	2-3 cm
Fresh Wt (mg/section)	11.2	22.2	30.2	31.3
Protein (µg/mg fr wt)	45.2	10.5	4.3	3.0
RNA (µg/mg fr wt)	310	114	90	110
DNA (µg/mg fr wt)	2.5	0.33	0.11	0.09
Total RNase Activity (µg RNA hydrolysed/hr)				
/mg fr wt	199	57	40	26
/µg protein	4.4	5.4	9.3	8.7
/µg DNA	80	173	364	289

Appendix VIII. Effects of the Nuclease Inhibitor Diethyl Pyrocarbonate (DEP) on the Extraction of Pea Epicotyl Polysomes.

Weeks and Marcus (1969) and Travis et al (1970) reported that an increase in the proportion of the ribosomal profile which is present as polysomes can be obtained by including DEP in the normal extraction medium for plant preparations. A number of such tests were carried out using pea epicotyls. First, it was confirmed that the pH of standard extraction buffers falls after adding DEP, presumably because this reagent decomposes in water to form ethanol and CO_2 (5). The pH could be maintained above 7.5 at 0 C for 1 hr, however, by using 50 - 100 mM HEPES-NaOH buffer (Appendix VIIIA). This pH was sufficiently high to prevent massive precipitation of particulate debris during fractionation.

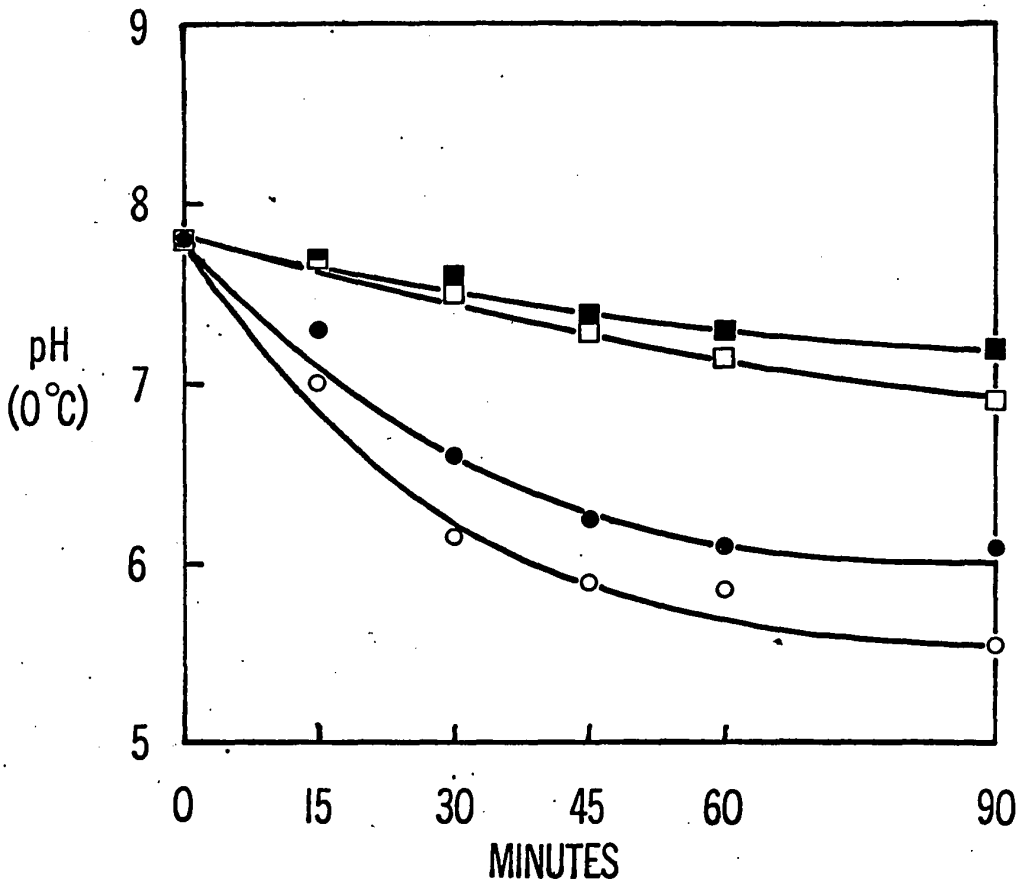
In the following experiment, polysomes were isolated from the pea epicotyl in the presence and absence of 1% (v/v) DEP. The standard extraction medium buffer was replaced by 100 mM HEPES-NaOH (pH 8.0 at 0 C). Appendix VIIIB shows the resulting profiles. The yield of ribosomes recovered in the 16,000 - 145,000g subfraction was increased by 60% after DEP treatment. The percentage of polysomes was increased by 10% (from 82 - 92%). It must be concluded that RNase activity in the preparation causes some degradation during the isolation procedure.

DEP could not be used in the main body of this study, of course, when RNase activity in ribosomes was to be assayed. It should be added that this reagent is not selective in its action against nucleases. It attacks tryptophan residues and

free amino groups and causes irreversible structural denaturation in proteins (17). It also causes ring-opening of adenine (9) and inhibits the amino acid incorporating ability of isolated polysomes (20,22).

Appendix VIIIA. Effect of DEP on pH of Extraction Buffer.

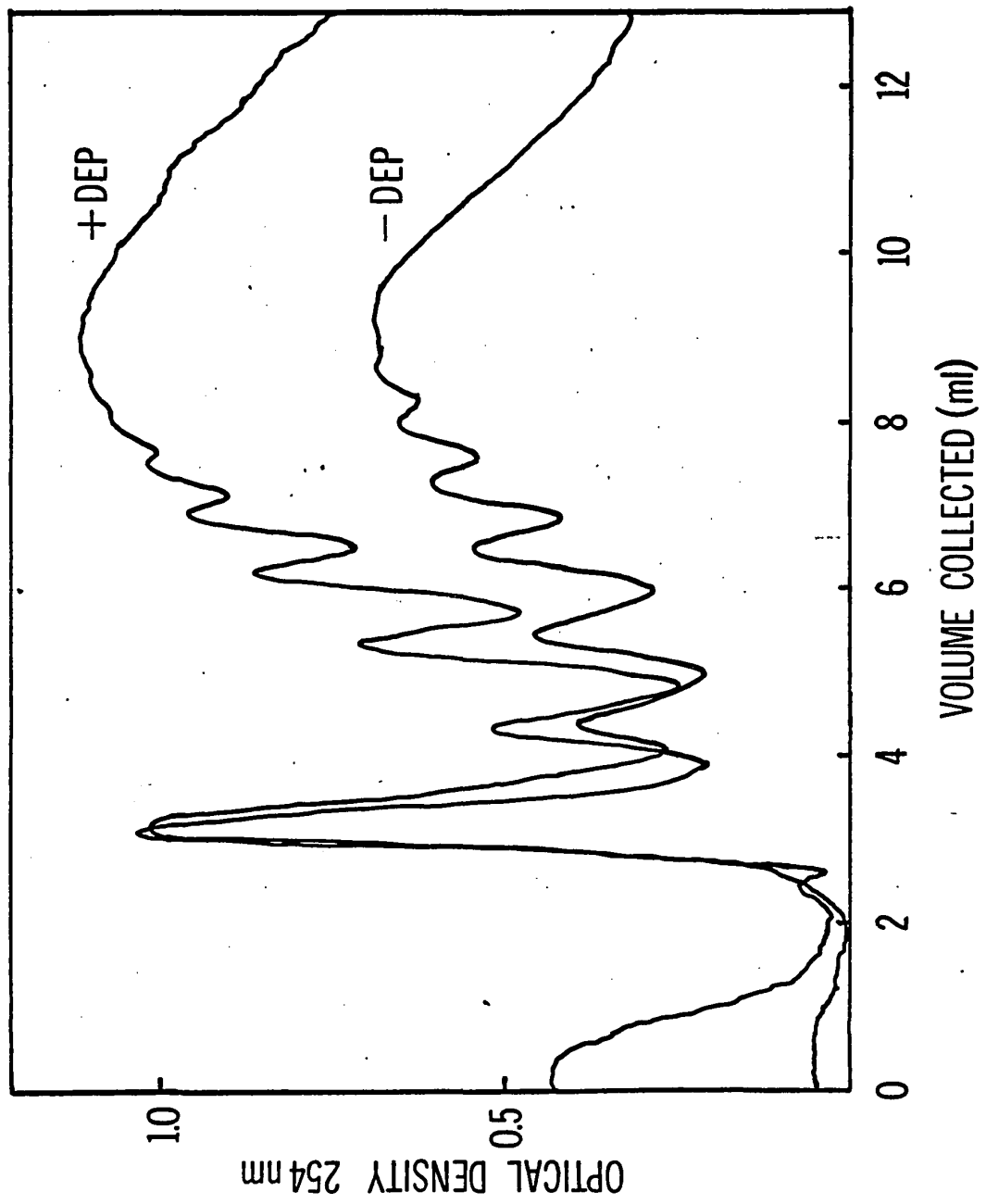
DEP [final concentration, 1% (v/v)] was added to different concentrations of Tris-HCl (pH 7.8 at 0 C) and HEPES-NaOH (pH 7.8 at 0 C) and the decrease in pH (at 0 C) was measured at 15 min intervals. 50 mM Tris-HCl (○); 100 mM Tris-CHl (●); 50 mM HEPES-NaOH (□); 100 mM HEPES-NaOH (■).



Appendix VIIIA. Effect of DEP on Extraction buffer

Appendix VIIIB. Effect of DEP on Yield and Percentage of Polysomes Extracted from Pea Epicotyls.

The apical tissue (top 10 to 12 mm) of 8 to 9-day old etiolated pea seedlings was collected in cold water, weighed and ground in 1 to 2 vols of a similar extraction medium to that reported in the main text (100 mM HEPES-NaOH (pH 7.7 at 22 C) was used in this experiment). DEP was added directly during extraction to give a final concentration of 1% (v/v), the rest of the extraction and preparation of polysomes was performed as described above. Total $O.D._{254\ nm}$ under profile: -DEP = 5.26, +DEP = 8.27; percentage of polysomes: -DEP = 82, +DEP = 92.



Appendix VIIIB. Effect of DEP on polysomes extracted from pea epicotyls.

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