The Degradation of Ribonucleic Acid in the Cotyledons of *Pisum arvense*

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1. A ribonuclease has been partially purified from the cotyledons of germinating seed of *Pisum arvense*. 2. The enzyme degrades ribopolynucleotides to adenosine 3'-phosphate, guanosine 3'-phosphate and the cyclic nucleotides cytidine 2',3'-phosphate and uridine 2',3'-phosphate; no resistant 'core' remains. 3. The activity of RNA-degrading enzymes in the cotyledons increases to a maximum during the first 5 days of germination, passes through a minimum around the eighth day, and thereafter increases again. 4. Ion-exchange chromatography of methanol-soluble extracts of cotyledons revealed the presence, amongst other components, of the 2', 3'- and 5'-phosphates of cytidine and uridine, the 3'- and 5'-phosphates of adenosine, and guanosine 5'-phosphate. 5. Seed soaked in a solution containing [³²P]orthophosphate gave a methanol-soluble fraction containing labelled nucleoside 5'-phosphates, but nucleoside 2'- and 3'-phosphates were not labelled. 6. It is believed that the nucleoside 2'- and 3'-phosphates arise by the action of ribonuclease on cotyledon RNA.

Barker & Hollinshead (1964) have shown that the quantity of RNA in the cotyledons of *Pisum arvense* falls during the first 2 weeks after germination and that little or no RNA synthesis takes place in the cotyledon during this period. The present work is concerned with the possible part played by ribonuclease in the degradation of RNA in the cotyledon during germination. Ribonucleases may fulfil a synthetic function (Barker, Montague, Moss & Parsons, 1957; Markham & Strominger, 1956; Barker & Douglas, 1960; Reddi, 1959), but are probably associated mainly with degradation (Dekker, 1960). Ribonucleases have been recognized in the storage organs of various plants such as the cotyledons of the pea (Barker & Douglas, 1960) and peanut (Cherry, 1963), and in corn seed (Wilson, 1963a,b; Ingle, 1963). This paper describes the partial purification of a ribonuclease present in the cotyledons of *P. arvense*. The products of the action of the enzyme on RNA have been studied and it is shown that the soluble nucleotides of the cotyledons include a number of components which are believed to arise by ribonuclease action.

MATERIALS AND METHODS

Seed of *P. arvense* (var. Minerva Maple) were a gift from Gartons of Warrington, Lancs. Germination and growth

were carried out as previously described (Barker & Hollinshead, 1964). Seed of New Zealand perennial ryegrass was a commercial specimen. Yeast RNA (British Drug Houses Ltd., Poole, Dorset) was purified by a modification of the method of Frisch-Niggemeyer & Reddi (1957). RNA from seed of *P. arvense* was prepared as described by Barker & Hollinshead (1964). Adenosine 2',3'-phosphate [nucleoside 2',3'-phosphates are cyclic phosphates; nucleoside 2'(3')-phosphates are mixtures of the 2'- and 3'-isomers] and guanosine 2',3'-phosphate were prepared by the method of Szer & Shugar (1963). Nucleoside triphosphates were purchased from Schwarz Bio-Research Inc. (Orangeburg, N.Y., U.S.A.). [³²P]Orthophosphate was obtained from The Radiochemical Centre (Amersham, Bucks.). Russell's viper venom was obtained in the freeze-dried state from Ross Allen's Reptile Institute, Silver Springs, Fla., U.S.A. All other materials were obtained from British Drug Houses Ltd., and were used without further purification.

Assay of ribonuclease. Ribonuclease activity was measured by a modification of the method of Dickman, Aroskar & Kropf (1956). The enzyme solution (0.5 ml.) was incubated at 37° for 15 min. with 0.3% (w/v) yeast RNA (0.5 ml.). The reaction was stopped by addition of 3 ml. of acetic acid-2-methylpropan-2-ol (1:2, v/v) with rapid mixing. The suspension was allowed to stand for 30 min. at 0° and the precipitate was then removed by centrifugation. A portion (1 ml.) of the supernatant was diluted to 6 ml. with water and the extinction was measured at 260 mμ. Control experiments were performed at the same time in which the acetic acid-butanol reagent was added before incubation. One unit of activity is taken as the quantity of enzyme producing a difference in extinction of 10 between experimental and control solutions under the above conditions.

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Extraction of ribonuclease. All operations were carried out at 0–4°. Cotyledons (0.75 g.) were homogenized in a glass homogenizer with either 0.2M-sodium citrate buffer over the range pH 3.6–6.6, or with 0.2M-tris-HCl buffer over the range pH 6.6–8.0; each buffer contained 0.01% (w/v) of gelatin. Cellular debris was removed by centrifugation at 9000g for 20 min. and rejected. The supernatant was centrifuged at 16000g for 30 min., yielding a pellet (P16). The supernatant from this pellet was centrifuged at 50000g for 45 min., yielding a further pellet (P50) and a supernatant which was further centrifuged at 100000g for 2 hr. This yielded a pellet (P100) and supernatant fraction (S). The particulate fractions P16, P50 and P100 were washed twice by resuspension in the same buffer as used for homogenization (5 ml.) and centrifugation and were finally resuspended in 5 ml. of the same buffer and subjected to ultrasonic vibration at 2° for 5 min. (Ultrasonic Disintegrator, Cabinet model 60; Measuring and Scientific Equipment Ltd.).

Partial purification of ribonuclease. The method used was similar to that of Holden & Pirie (1955a). All operations were carried out at 0–4°. Peas (500 g.) were germinated for 4 days and the cotyledons were homogenized in batches in a Waring Blender with a total of 500 ml. of 0.1M-sodium citrate buffer, pH 5.6. The homogenate was squeezed through muslin and the filtrate was centrifuged at 3000g for 20 min. The supernatant was brought to pH 4.5 by addition of N-HCl, allowed to stand for 3 hr. and the precipitate was removed by centrifugation at 8000g for 20 min. and discarded. The supernatant was brought to pH 5.5 by addition of N-NaOH, solid $(\text{NH}_4)_2\text{SO}_4$ was added to 35% saturation and the solution was allowed to stand overnight. The precipitate was removed by centrifuging at 8000g for 30 min. and discarded. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to a concentration of 85% saturation. After standing overnight, the precipitate was collected by centrifugation as above and dissolved in 100 ml. of 0.1M-sodium citrate buffer, pH 5.6. A small insoluble residue was removed by centrifugation and the solution was dialysed twice (12 hr. each) against 5 l. of distilled water, the dialysis tubing being changed after 12 hr. to avoid rupture. Fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$ as described above was repeated once more and the precipitate obtained at 85% saturation was dissolved in 50 ml. of 0.1M-sodium citrate buffer, pH 5.6. A small precipitate was removed as before and the supernatant was dialysed against 3 l. of distilled water. The ribonuclease activity of the solution was determined as described above; the protein content was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), by using crystalline bovine plasma albumin as standard.

Extraction and separation of nucleotides. Cotyledon tissue from pea seeds (15 g. samples), freed from embryos and testas, was homogenized in an MSE homogenizer for 5 min. at 0° with methanol (250 ml.) and the homogenate centrifuged at 2000g for 20 min. The residue was extracted by homogenization at 0° for 5 min. with two 125 ml. portions of 0.05N-formic acid in methanol and the homogenate centrifuged as before. Methanol was removed from the combined supernatants under reduced pressure at 0° and the remaining yellow suspension (about 5 ml.) was clarified by filtration through Celite. The solution was extracted twice with an equal volume of ether and the aqueous phase

was again filtered through Celite. The pale-yellow filtrate was adjusted to pH 8 by addition of N-KOH and clarified by centrifugation. In some experiments the resulting solution was loaded directly on to an ion-exchange chromatographic column. In other experiments, the solution was purified by treatment with charcoal by the method of R. C. Hignett (personal communication). Norite charcoal (2 g.) was activated immediately before use by holding under reduced pressure in 6N-HCl (20 ml.) for 3 hr. The material was repeatedly washed with water by decantation until the washings were at pH 5. The charcoal was then suspended in a volume of 0.1N-HCl to give a solution containing 10 mg. of Norite/ml. Optimum adsorption of nucleotides was achieved by using 1 ml. of Norite suspension for approx. 23 $E_{260\text{m}\mu}$ units of nucleotides and maximum recovery was obtained under these conditions.

The nucleotide-containing solution was added to the appropriate volume of Norite suspension. The mixture was allowed to stand at room temperature for 10 min. and was then poured on to a layer (1 cm. thick) of Celite, previously washed with 6N-HCl and water, and supported on a sintered-glass disk. The column of charcoal was washed with water until the eluate was at pH 5 and the washings were discarded. The column was then eluted with a solution containing 10% (v/v) of 0.5N- NH_3 soln., 50% (v/v) of ethanol and 40% (v/v) of water (10 ml. of the solution for each millilitre of Norite suspension). This operation should take at least 1 hr. The eluate was evaporated to about 2 ml. under reduced pressure and adjusted to pH 8 by addition of N-KOH.

Ion-exchange chromatography. In different experiments, methanol-soluble nucleotides were separated by ion-exchange chromatography on either Dowex 1 resin, by the method of Hurlbert, Schmitz, Brumm & Potter (1954), or on Dowex 21K. With the former, the dimensions of the column were 12 cm. $\times$ 1 cm. and the resin had a particle size of 200–400 mesh. Gradient elution was carried out with a 250 ml. mixing vessel initially, containing water into which was passed successively 500 ml. of 2N-formic acid, 500 ml. of 4N-formic acid and 500 ml. of 0.8M-ammonium formate in 4N-formic acid. The rate of flow was 0.4 ml./min. Dowex 21K was used in the formate form and had a particle size of 50–100 mesh. The dimensions of the column were 70 cm. $\times$ 1 cm. and the rate of flow was 0.35 ml./min. Gradient elution was carried out as described above, with a 500 ml. mixing vessel and 1 l. of each eluting solution. In each case, the eluate from the column was collected in 5 ml. fractions and $E_{260\text{m}\mu}$ of each fraction was measured. Radioactivity was measured as described by Barker & Hollinshead (1964).

The fractions corresponding to individual peaks were pooled, and the solvent was removed under reduced pressure at 0°. More labile fractions were freeze-dried. Ammonium ions were removed from fractions containing ammonium formate by use of Amberlite IR-120 resin. Each fraction was examined as described below.

Paper electrophoresis. This was carried out on strips of acid-washed Whatman no. 1 chromatography paper, 60 cm. long, by the method of Markham (1955). The buffer, pH 3.5, contained acetic acid (92 ml.) and redistilled pyridine (10 ml.), the mixture being diluted to 1600 ml. with water. A potential of 2000 v was maintained, giving a gradient of approx. 40 v/cm. during a standard run of 75 min. Relative electrophoretic mobilities are expressed

as the distance travelled towards the anode under these conditions.

Paper chromatography. Compounds were separated in the following solvent systems: (1) isobutyric acid-aq. NH_3 soln. (sp.gr. 0.880)-water (66:1:33, by vol.) (Pabst Laboratories, 1961); (2) 7.7% (w/v) ammonium acetate (pH 7.5)-95% (v/v) ethanol (3:7, v/v) (Pabst Laboratories, 1961); (3) isoamyl alcohol-5% (w/v) disodium hydrogen phosphate (1:1, v/v) (Cohn & Carter, 1950); (4) 3.8% (w/v) ammonium carbonate-propan-2-ol (1:3, v/v) (Stockx, 1958); (5) propan-1-ol-aq. NH_3 soln. (sp.gr. 0.880)-water (6:3:1, by vol.) (Hanes & Isherwood, 1949); (6) butan-1-ol-propionic acid-water (75:36:49, by vol.) (Tyszkiewicz, 1962). Whatman no. 52 paper was used throughout and was washed with acid to remove ultraviolet-absorbing material; the descending method of chromatography was used except with solvent systems (2) and (3). Ultraviolet-absorbing material was detected by viewing with a Hanovia Chromatolite and by the method of Markham & Smith (1949); radioactive materials were located by radioautography. Appropriate authentic compounds were chromatographed on the same paper as unknown materials.

Ninhydrin reagent. Chromatograms or electrophoretograms were dipped in a 0.25% (w/v) solution of ninhydrin in acetone, dried and heated at 100° for 5 min.

Hydrolysis by 3'-nucleotidase. Rye-grass seed, *Lolium perenne* L. (500 g.), was germinated at 26° for 3 days on filter paper moistened with $10\text{ }\mu\text{M}$ -gibberellic acid. The enzyme was extracted from the seed by the method of Shuster & Kaplan (1955) with the following modification. The first ethanol precipitate was centrifuged after standing for 3 hr. at -5° and was found to contain the bulk of the 3'-nucleotidase free from 2'- or 5'-nucleotidase. No further purification was attempted. The enzyme was stored at 0° and retained its activity for many months.

Hydrolysis was performed by incubating the nucleotide (2-3 mg.) in 0.5 ml. of solution brought to pH 7.5 by addition of aq. 0.1 N- NH_3 soln. with 0.1 ml. of enzyme solution (approx. 5 mg./ml.) and 0.01 M-sodium phosphate or 0.01 M-ammonium hydrogen carbonate buffer, pH 7.5 (0.5 ml.), for 1 hr. at 37° . The digest was evaporated under reduced pressure to small volume and chromatographed in solvent (1).

Hydrolysis by 5'-nucleotidase. The venom of Russell's viper was used as supplied. Hydrolysis was performed by incubating the nucleotide (2-3 mg.) in 0.5 ml. of solution brought to pH 9.0 by addition of aq. 0.1 N- NH_3 soln. with 0.1 ml. of enzyme solution (approx. 5 mg./ml.) in 0.01 M-tris-HCl buffer, pH 9.0 (0.5 ml.), for 3 hr. at 37° . The digest was evaporated as described above and chromatographed in solvent (1).

RESULTS

Distribution of ribonuclease activity in different cellular fractions. The following results were obtained with extracts prepared from cotyledons after 3 days' germination. Fractions P16 and P50 of the tissue extract showed no significant activity at any pH between 3.5 and 8.0. Fraction P100 showed apparent activity between pH 5.2 and 6.8 (Fig. 1, curve I). However, it was observed that in the assay procedure, after centrifugation of

Fig. 1. Rate of degradation of RNA as a function of pH by fraction P100 (Δ) and fraction S ($\bullet$).

Table 1. Changes in ribonuclease activity of cotyledons during germination

Seeds were germinated for varying times and ribonuclease activity in the soluble fraction of the cotyledons was determined as described in the text.

Time of germination (days)	Activity	
	Units/g. of fresh tissue	Units/g. of dry tissue
1	24	46
2	175	403
3	210	409
4	250	585
5	267	693
6	205	559
7	150	465
8	130	382
9	303	740
10	424	885
12	440	843
14	617	1100

acid-precipitable material, a very faint opalescence remained in those incubation mixtures which showed apparent enzymic activity. In each case, addition of an equal volume of ethanol and centrifugation for 20 min. at $10000g$ removed the opalescence and gave a supernatant having the same $E_{260\text{ m}\mu}$ as a blank to which the precipitating reagent was added at zero time. It is therefore concluded that the values shown in Fig. 1, curve I, represent an artifact and that fraction P100 is devoid of ribonuclease activity. No opalescence was observed in enzyme assays with fraction S, and activity exhibiting a broad peak at about pH 5.6 was observed (Fig. 1, curve II).

Changes in ribonuclease activity during germination. Seeds were germinated for varying times and the root and shoot were removed. Extracts were prepared from the cotyledons as described in the

Materials and Methods section by using buffer at pH 5.6, and ribonuclease activity was determined in fraction S. The results are shown in Table 1.

Action of cotyledon ribonuclease on polynucleotides. The partially purified enzyme solution, prepared as described in the Materials and Methods section, contained 200 units/ml. and 70 units/mg. of protein. Experiments were carried out with the following substrates: (i) purified yeast RNA, (ii) RNA 'core' remaining after digestion of yeast RNA with pancreatic ribonuclease, (iii) RNA from seed of *P. arvense*, (iv) polyadenylic acid, (v) polycytidylic acid, (vi) polyuridylic acid and (vii) polyinosinic acid. The polynucleotide (2mg.) was dissolved in 0.2ml. of 0.1M-sodium citrate buffer, pH 5.6, and incubated at 37° for 17hr. with 0.1ml. of enzyme solution. The incubation mixture was then chromatographed on paper with solvents (1) and (4). With substrates (i), (ii) and (iii), strong ultraviolet-absorbing spots were observed corresponding to cytidine 2',3'-phosphate, uridine 2',3'-phosphate, adenosine 3'-phosphate, guanosine 3'-phosphate, adenosine and guanosine. Very little material appeared on the starting line. Substrates (iv), (v) and (vi) were converted completely into materials having R_f values corresponding to adenosine 2'(3')-phosphate, cytidine 2',3'-phosphate and uridine 2',3'-phosphate respectively. Substrate (vii) gave rise to inosine 2'(3')-phosphate together with traces of inosine and hypoxanthine. In all cases, control experiments in which the polynucleotide was incubated at pH 5.6 without the addition of enzyme showed only material on the starting line.

Action of cotyledon ribonuclease on cyclic nucleotides. The nucleoside 2',3'-phosphate (2mg.) was dissolved in 0.2ml. of 0.1M-sodium citrate buffer, pH 5.6, and incubated at 37° with 0.01ml. of enzyme solution (2 units) for 17hr. The solutions were then chromatographed alongside controls to which no enzyme had been added, by using solvent (4), which causes little hydrolysis of cyclic phosphates (Stockx, 1958). Cytidine 2',3'-phosphate and uridine 2',3'-phosphate were unchanged by incubation with the enzyme; adenosine 2',3'-phosphate and guanosine 2',3'-phosphate gave rise to the corresponding nucleoside 2'(3')-phosphates together with traces of adenosine and guanosine.

Incorporation of [32 P]orthophosphate into the methanol-soluble fraction of cotyledons. Peas (18g.) were soaked for 17hr. in 0.01M- $\text{KH}_2^{32}\text{PO}_4$ (35ml.) and germinated for 3 days. The methanol-soluble nucleotides were extracted from the cotyledons and chromatographed on Dowex 1 ion-exchange resin as described in the Materials and Methods section without treatment with charcoal. Fig. 2 shows the extinction at 260m μ and the total radioactivity of each fraction. Material from each peak

Fig. 2. Chromatography of methanol extract of cotyledon tissue of *P. arvense* on Dowex 1. ●, E_{260} ; ▲, counts/min.

was submitted to electrophoresis and paper chromatography.

In preliminary experiments with seed that had not been treated with radioactive phosphate, it was found that peaks 1 and 2 consisted of a complex mixture containing purine bases and ultraviolet-absorbing material which also gave a positive reaction with ninhydrin. These peaks were not investigated further. Peak 4 yielded material exhibiting material absorbing at 257m μ and 290m μ , but no radioactivity. It was not investigated further. It may correspond to the unidentified component 5b obtained by Brown (1962) from perchloric acid extracts of mature pea seed.

Ultraviolet-absorbing components from the other peaks which corresponded to known nucleotides on electrophoresis and paper chromatography are shown in Table 2. The chromatograms were examined by radioautography and those components exhibiting no radioactivity are indicated in the Table.

Approximately 80% of the radioactivity of the methanol extract was associated with peaks A, B and C (Fig. 2), which were separately chromatographed in solvents (5) and (6). With the exception of material from peak C, which corresponded with ultraviolet-absorbing peak 6, and contained ADP, these peaks showed no ultraviolet absorption. All three yielded a series of spots on chromatograms which gave positive tests with the periodate-permanganate spray reagent for carbohydrates (Lemieux & Bauer, 1954) together with some inorganic phosphate. These radioactive peaks were believed to contain principally carbohydrate phosphates and were not investigated further.

Table 2. *Components identified in methanol-soluble fraction of cotyledons after chromatography on Dowex 1*

Material from peaks shown in Fig. 2 was submitted to paper electrophoresis and paper chromatography as described in the text. Components were identified by comparison of mobilities and R_F values with those of authentic samples and by methods indicated under Notes.

Peak	Component	Electrophoretic migration (cm.)	R_F			Notes
			Solvent (1)	Solvent (2)	Solvent (3)	
3	Cytidine 5'-phosphate	2.6	0.26	0.28	—	Insufficient material for further investigation
	Adenosine 5'-phosphate	4.8	0.51	0.27	—	Hydrolysed by 5'-nucleotidase to adenosine (R_F 0.70 in solvent 1)
	Cytidine 3'-phosphate	3.0	0.20	—	0.84	This material was not radioactive. It was hydrolysed by 3'-nucleotidase to cytidine (R_F 0.52 in solvent 1; R_F 0.74 in solvent 3); R_F of the material was unchanged after treatment with 5'-nucleotidase
5	Guanosine 5'-phosphate	8.9	0.13	0.12	0.81	Hydrolysed by 5'-nucleotidase to guanosine (R_F 0.25 in solvent 1). R_F unchanged after treatment with 3'-nucleotidase
	Uridine 5'-phosphate	11.8	0.13	0.22	0.85	Hydrolysed by 5'-nucleotidase to uridine (R_F 0.25 in solvent 1)
	Uridine 2'(3')-phosphate	12.0	0.17	0.30	—	This material was not radioactive. Treatment with 3'-nucleotidase and chromatography in solvent (1) gave one component having the same R_F as the original material and uridine (R_F 0.29)
6	ADP	13.6	0.24	0.20	—	Treatment with 5'-nucleotidase gave a component corresponding to adenosine (R_F 0.70 in solvent 1)
7	Uridine 5'-phosphate	11.8	0.14	0.22	—	Hydrolysed by 5'-nucleotidase to uridine (R_F 0.25 in solvent 1)
	Uridine diphosphate glucose	18.0	0.03	0.48	—	Treatment with 5'-nucleotidase gave a component corresponding to uridine (R_F 0.29 in solvent 1)
	UDP	21.5	0.06	0.18	—	Treatment with 5'-nucleotidase gave a component corresponding to uridine (R_F 0.29 in solvent 1)
8	ATP	18.5	0.13	0.12	—	Treatment with 5'-nucleotidase gave a component corresponding to adenosine (R_F 0.70 in solvent 1)
	ADP	13.6	0.2	0.20	—	
	AMP	4.8	—	0.27	—	

Chromatography of methanol extracts from cotyledons of unripe seed, ripe seed and seed taken after varying times of germination revealed similar ultraviolet-absorbing peaks, the relative sizes of which varied with the stage of development; the unidentified material in peak 4 was, however, present only in cotyledons from germinating seed. Chromatography of the methanol extracts on Dowex 1 served to demonstrate the absence of isotopically labelled phosphorus from nucleotides carrying a 2'- or 3'-phosphate residue. It did not, however, allow for the identification of all the

Fig. 3. Chromatography of nucleotides on Dowex 21K. Experimental details are given in the text.

Table 3. *Separation of nucleotides by chromatography on Dowex 21K*

Material from peaks shown in Fig. 3 was submitted to paper electrophoresis and paper chromatography as described in the text, authentic samples of the compounds being run simultaneously on the same paper.

Peak	Components	Electrophoretic migration (cm.)	R_F	
			Solvent (1)	Solvent (2)
A	Cytidine 5'-phosphate	2.9	0.26	—
B	Cytidine 2'-phosphate	3.2	0.29	—
	Cytidine 3'-phosphate	3.2	0.20	—
C	Adenosine 5'-phosphate	4.8	0.51	—
	Adenosine 3'-phosphate	5.9	0.53	—
D	Adenosine 2'-phosphate	5.9	0.58	—
E	Guanosine 5'-phosphate	8.9	0.13	0.12
F	Guanosine 2'(3')-phosphate	9.5	0.13	0.16
G	Inosine 5'-phosphate	10.9	0.13	0.14
H	Uridine 5'-phosphate	11.8	0.14	0.22
	Uridine 2'(3')-phosphate	12.2	0.17	0.3

nucleoside 2'- and 3'-phosphates and this was attempted by using Dowex 21K.

Chromatography of nucleotides on Dowex 21K. A solution (5ml.) containing approximately 2.5mg. each of adenosine 2', 3'- and 5'-phosphate, guanosine 2', 3'- and 5'-phosphate, cytidine 2', 3'- and 5'-phosphate and uridine 2', 3'- and 5'-phosphate was treated with charcoal as described in the Materials and Methods section and chromatographed on a column of Dowex 21K. The elution diagram is shown in Fig. 3. Material from each peak was submitted to electrophoresis and paper chromatography. The identities of compounds present in each peak are indicated in Table 3.

Chromatography of methanol-soluble extract of cotyledons on Dowex 21K. The methanol-soluble extract was prepared from cotyledon tissue, taken after germination for 2.5 days, as described above. After treatment with charcoal the material was chromatographed on a column of Dowex 21K. The elution diagram is shown in Fig. 4. Material from individual peaks was examined by paper electrophoresis and paper chromatography. Peaks 1 and 2 were not completely resolved. Material in peak 6 corresponded to no known nucleotide and was not identified. Components identified in other peaks are shown in Table 4.

DISCUSSION

Matsushita & Ibuki (1960) and Lyndon (1966) have reported the presence of active ribonucleases associated with cytoplasmic particles of pea shoots and roots. In the present experiments with cotyledons, the apparent activity in the ribosomal fraction was found to be due to an artifact. In confirmation of the nature of the tissue fraction used, the presence of particles having characteristics

of ribosomes was demonstrated by ultracentrifugation and electron microscopy (Barker & Hollinshead, 1965). These studies showed that cotyledon ribosomes differ in stability from shoot ribosomes and a difference in enzymic content is therefore not unexpected. Wilson & Shannon (1963) have suggested that the apparent presence of ribonucleases in the subcellular particles of the storage tissues of plants is due to contamination with enzymes present in the supernatant fraction, but in the present experiments ribosomal pellets were washed twice before examination for enzyme activity. Lett, Takahashi & Birnstiel (1963) also found that incubation of ribosomal preparations from peas during the assay of ribonuclease results in the appearance of turbidity. This phenomenon may be related to the observations in the present experiments, and it is possible that the apparent ribonuclease activity reported by these authors may also be due to an artifact. In the present experiments turbidity which developed during the assay was removed under conditions that would not be expected to precipitate mononucleotides and so obscure their formation during the incubation. The nature of the material causing the turbidity is not known and therefore, although the possibility of the presence of oligonucleotides as products of partial enzymic degradation cannot be excluded, it is considered that the results indicate the presence of an active ribonuclease unequivocally only in the supernatant fraction of the cotyledons.

In agreement with previous observations on pea ribonuclease (Holden & Pirie, 1955*a,b*; Pierpoint, 1956; Markham & Strominger, 1956), the present results indicate that the ribonuclease from pea cotyledons degrades RNA completely to mononucleotides without leaving a resistant 'core'.

Table 4. *Components identified in methanol-soluble fraction of cotyledons after chromatography on Dowex 21K*

Material from peaks shown in Fig. 4 was submitted to paper electrophoresis and paper chromatography as described in the text. Components were identified by comparison of mobilities and R_F values with those of authentic samples and by methods indicated under Notes.

Peak	Components	Electrophoretic migration (cm.)	R_F		Notes
			Solvent (1)	Solvent (2)	
1	Cytidine 5'-phosphate	2.9	0.26	—	Hydrolysed by 5'-nucleotidase to cytidine (R_F 0.52 in solvent 1)
	Cytidine 2'-phosphate	3.2	0.29	—	R_F unchanged after treatment with either 5'-nucleotidase or 3'-nucleotidase
	Cytidine 3'-phosphate	3.2	0.20	—	Hydrolysed by 3'-nucleotidase to cytidine (R_F 0.52 in solvent 1)
2	Cytidine 2'-phosphate	3.2	0.29	—	R_F unchanged after treatment with either 5'-nucleotidase or 3'-nucleotidase
	Cytidine 3'-phosphate	3.2	0.20	—	Hydrolysed by 3'-nucleotidase to cytidine (R_F 0.52 in solvent 1)
3	NAD	3.9	0.29	—	
4	Adenosine 5'-phosphate	4.8	0.51	—	Hydrolysed by 5'-nucleotidase to adenosine (R_F 0.70 in solvent 1)
5	Adenosine 3'-phosphate	5.3	0.53	—	Hydrolysed by 3'-nucleotidase to adenosine (R_F 0.70 in solvent 1)
7	Guanosine 5'-phosphate	—	0.13	—	The material was present in very small quantity contaminated with unidentified material. Its presence in this peak is believed to be due to contamination with peak 8
8	Guanosine 5'-phosphate	8.9	0.13	0.12	Hydrolysed by 5'-nucleotidase to guanosine (R_F 0.25 in solvent 1)
9	Inosine 5'-phosphate	10.9	0.13	0.14	
10	Uridine 5'-phosphate	11.8	—	0.22	Hydrolysed by 5'-nucleotidase to uridine (R_F 0.29 in solvent 1)
	Uridine 2'(3')-phosphate	12.2	—	0.30	Treatment with 3'-nucleotidase and chromatography in solvent (1) gave one component having the same R_F as the original material and uridine (R_F 0.29)
	NADP	10.1	—	0.06

Fig. 4. Chromatography of methanol extract of cotyledon tissue of *P. arvense* on Dowex 21K. Experimental details are given in the text.

This was observed with all the polynucleotide substrates used, including the 'core' of yeast RNA, which is resistant to crystalline pancreatic ribonuclease. The polynucleotides of natural origin all

gave rise to adenosine 2'(3')-phosphate, guanosine 2'(3')-phosphate, cytidine 2',3'-phosphate and uridine 2',3'-phosphate, together with traces of degradation products. This indicates that the enzyme attacks all 5'-phosphate ester linkages in the polynucleotides. This conclusion is also in agreement with results obtained with enzymically synthesized polyadenylic acid, polyinosinic acid, polycytidylic acid and polyuridylic acid. Pyrimidine-containing nucleotide residues are converted into cytidine or uridine 2',3'-phosphate and these cyclic phosphates are not hydrolysed further by the enzyme. It would appear likely that cyclic phosphates are first formed from purine nucleotide residues but no evidence for their intermediate formation was obtained. Incubation of purine nucleoside cyclic phosphates with the enzyme results in their complete conversion into mixtures of nucleoside 2'- and 3'-phosphates whereas in the absence of the enzyme they remain largely un-

changed. It is probable that the enzyme is a ribonucleate nucleotido-2'-transferase (cyclizing) (EC 2.7.7.17), but further investigation would be required to confirm this.

The ultraviolet-absorbing material present in methanol extracts of pea cotyledons consists of a mixture far more complex than that obtained by Cherry (1962) from peanut cotyledons. The results presented are similar to those obtained by Brown (1962) with perchloric acid extracts of pea seed. However, in addition to the nucleoside 5'-phosphates, -diphosphates and -triphosphates, we have observed the presence of nucleoside 2'- and 3'-phosphates which were not recognized by Brown. Such compounds have previously been reported in wheat, barley and oat plants (Bergkvist, 1956, 1957) and in *Phaseolus vulgaris* (Šebesta & Šorm, 1959), but, in contrast, in the present work no guanosine 2'- or 3'-phosphate was recognized although it was demonstrated that authentic samples were separable by the technique used. In view of the high activity of ribonuclease in the cotyledons after 2.5 days' germination, and the nature of the products of the action of the enzyme on ribopolynucleotides, it appears probable that the nucleoside 2'- and 3'-phosphates present in the methanol extracts arise by degradation of RNA by ribonuclease in the cotyledon. Whereas phosphate residues attached to position 5 of the ribose residues of the methanol-soluble nucleotides became labelled by [³²P]orthophosphate, no radioactivity was present in the nucleoside 2'- or 3'-phosphates identified. This is in agreement with the above hypothesis with regard to the origin of the 2'- and 3'-phosphates since it has been shown (Barker & Hollinshead, 1964) that the RNA of cotyledons does not become labelled by treatment of the seed with [³²P]orthophosphate. It is concluded that the ribonuclease in the soluble fraction of the cotyledons is functionally active during germination and that it is connected, at least in part, with degradation of RNA in the cotyledon. It is not possible at present to account for the absence of guanosine 2'- or 3'-phosphate from methanol-soluble extracts of cotyledons. This does not appear to be due to the specificity of the cotyledon ribonuclease. It may indicate some function of guanine nucleotides in the processes of germination, and in this connexion it is significant that acid hydrolysis of the material present in peak 4 (Fig. 2) yielded guanine. The presence of most of the radioactivity in chromatographic components showing no absorption in the ultraviolet region and reacting with the periodate-permanganate spray reagent suggests, not unexpectedly, that the phosphorus metabolism of germinating pea cotyledons is associated largely with the formation of phosphate esters of carbohydrates.

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