Nucleic Acid and Protein Synthesis Associated with the Induction of Nitrate Reductase Activity in Radish Cotyledons

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1. RNA and protein synthesis was studied during the incubation of excised radish cotyledons in nitrate, conditions that induced nitrate reductase activity in the tissue. 2. Synthesis of total RNA and protein, as measured by the incorporation of radioactive precursor, was significantly stimulated in the presence of nitrate (compared with chloride control), but was decreased in the presence of ammonium nitrate, which induced higher enzyme activity. 3. Synthesis of RNA and protein was required for induction of enzyme activity, as determined by using the inhibitors actinomycin D, puromycin and cycloheximide. 4. On the basis of 5-fluorouracil inhibition, the synthesis of only DNA-like RNA was required for induction, but no differences, either quantitative or qualitative, were observed in DNA-like RNA synthesis in the presence or absence of induction. 5. A 100-fold purification of the nitrate reductase activity showed no increase in nitrate reductase protein, nor any increased incorporation of radioactive precursor into nitrate reductase protein in the induced versus the control system. Such results suggested that the protein synthesis required for induction may be for a protein other than nitrate reductase.

Growth and development of plant tissues is thought to proceed by, or at least be accompanied by, changes in the complement of enzyme activities within the cell (Heyes & Brown, 1965). Consequently an understanding of the mechanisms that regulate enzyme activity is essential for the study of growth and development. Nitrate reductase has been a convenient enzyme to use in such regulatory studies, since its activity fluctuates diurnally (Hageman, Flesher & Gitter, 1961) and with phases of morphological development during growth (Zieserl, Rivenbark & Hageman, 1963) and it may be controlled experimentally by the external concentration of nitrate in the nutrient medium (Hageman & Flesher, 1960). Similar induction of activity may be achieved by using excised tissues (Beevers, Schrader, Flesher & Hageman, 1965). Further, it has been shown that incubation of excised radish cotyledons in nitrate induces specifically nitrate reductase, and indirectly nitrite reductase, without affecting the general metabolism of the tissue (Ingle, Joy & Hageman, 1966). The nitrate reductase activity of the cotyledons was not inhibited by any of the normal end products of nitrate assimilation, indicating that regulation of this particular enzyme activity by feedback inhibition may be of little importance (Ingle *et al.* 1966). The use of inhibitors such as azaguanine, puromycin and actinomycin D has indicated that

synthesis of both protein and RNA are required for the induction of nitrate reductase activity (Beevers *et al.* 1965; Ingle *et al.* 1966). This suggests that the induction is regulated at the transcriptional level of messenger RNA synthesis. However, no detailed information on the action of these inhibitors on protein and RNA synthesis is available, and there is as yet (Beevers *et al.* 1965) no evidence that new synthesis of nitrate reductase protein occurs during induction.

The present investigations were undertaken to study in greater detail certain aspects of protein and nucleic acid synthesis associated with the induction, by nitrate, of nitrate reductase in cotyledons excised from radish seedlings.

MATERIALS AND METHODS

Plant material. Radish seedlings (*Raphanus sativus* L. var. Cherrybelle) were grown under nitrogen-deficient conditions for 5 days, after which the cotyledons were excised and incubated as described by Ingle *et al.* (1966).

Measurement of RNA and protein synthesis and determination of nitrate reductase activity. Duplicate samples of cotyledons (15 pairs) were incubated in a medium containing 1 mM-potassium phosphate buffer, pH 5.5, and inhibitor if required, in a final volume of 7.5 ml. Incubation was at 30° with constant shaking in the dark. After 1 hr. inducer substrate (13 mM- NO_3^- or NH_4NO_3 with Cl^- as the control anion) and 10 μC of [^3H]uridine (3 C/m-mole) plus 0.3 μC of

[^{14}C]leucine (150 mc/m-mole) were added, and the incubation was continued for 3 hr. The cotyledons were removed from the medium, washed thoroughly with ice-cold water and homogenized in 10 ml. of 10 mM-potassium phosphate-10 mM-EDTA-5 mM-cysteine, pH 8.2, for 30 sec. in a VirTis homogenizer at medium speed. Four 2 ml. samples of the homogenate were immediately removed, two into 2 ml. of 10% (w/v) perchloric acid and two into 2 ml. of 10% (w/v) trichloroacetic acid for RNA and protein determinations respectively. The remaining homogenate was filtered through four layers of cheesecloth and centrifuged at 20 000g for 15 min., and the nitrate reductase activity in the supernatant was assayed as described by Hageman & Flesher (1960) and expressed as milliunits (μmoles of nitrite produced/min.).

The RNA samples were centrifuged (1500g for 10 min.) and the pellets washed briefly with cold 5% (w/v) perchloric acid before being defatted by two treatments with ethanol-ether-chloroform (2:1:1, by vol.). The residue was dissolved in 1 ml. of 0.3 N-KOH, and the RNA was hydrolysed for 16 hr. at 37°. The solution was acidified with perchloric acid, cooled to 0° for 15 min. and centrifuged to remove the DNA and protein. The supernatant was neutralized with KOH, cooled and centrifuged. Portions of the supernatant were used to determine the RNA content by measurement of E_{260} , and ^3H radioactivity by counting in a Packard liquid-scintillation spectrometer (50-150 window, 40% gain), with 0.4 ml. of sample/15 ml. of scintillator [385 ml. of toluene, 385 ml. of dioxan, 235 ml. of methanol, 81 g. of naphthalene, 5 g. of 2,5-diphenyloxazole and 87 mg. of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene/1000 ml.].

The protein samples were centrifuged, washed once with cold 5% trichloroacetic acid and defatted by two extractions with ethanol-ether-chloroform. The residues were dissolved in 2 ml. of 1 N-NaOH and the RNA was partially hydrolysed for 1 hr. at 37°. The protein and DNA were precipitated with trichloroacetic acid (5% final concn.), washed once with 5% trichloroacetic acid and dissolved in 2 ml. of aq. 2 N-NH₃ soln. Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin fraction V as standard. The ^{14}C radioactivity was measured by plating portions of the solution on to planchets, which were dried and counted on a Beckman Low Beta II instrument. Under these conditions of isotope input, of chemical fractionation of the protein and RNA and of differential counting of the ^3H and ^{14}C , contamination of the [^{14}C]protein radioactivity with ^3H was less than 1%, and ^{14}C contamination in the [^3H]RNA determination was less than 5%.

Preparation and fractionation of nucleic acids. Total nucleic acid was prepared by either the phenol-sodium dodecyl sulphate or the naphthalenedisulphonate-phenol-tri-isopropyl-naphthalenesulphonate-*p*-aminosalicylate method as described by Loening & Ingle (1967). Both methods, and several modifications of these methods, gave identical RNA preparations, but differed in the degree of breakage of the DNA (Loening & Ingle, 1967). The nucleic acids were fractionated by MAK* column chromatography (Ingle, Key & Holm, 1965) and by gel electrophoresis (Loening & Ingle, 1967). The RNA contents of fractions from the MAK column were determined by measuring

E_{260} . When [^{32}P]phosphate was used as a nucleic acid precursor, radioactivity was determined by drying portions of the fractions on planchets, which were counted with a Nuclear-Chicago gas-flow counter. When ^3H - or ^{14}C -labelled precursors were used, the fractions were acidified with trichloroacetic acid (5% final concn.), filtered on to Millipore membranes, washed with 5% trichloroacetic acid, dried and counted in a Packard liquid-scintillation spectrometer.

Gels were scanned at 265 μ on a Joyce-Loebel Chromoscan.

Double labelling of the nucleic acids. In double-labelling experiments, designed to show whether differences in RNA synthesis occurred between induced and non-induced tissue, one sample of 30 pairs of cotyledons was incubated in the presence of 10 mM-Cl⁻ and [^3H]uridine (150 μC , 2 c/m-mole) plus [^3H]adenosine (150 μC , 4 c/m-mole), and an identical sample was incubated in 10 mM-NO₃⁻ and [^{14}C]uridine (15 μC , 39 mc/m-mole) and [^{14}C]adenosine (15 μC , 41.6 mc/m-mole). The concentrations of the uridine and adenosine in both the ^3H - and ^{14}C -labelled incubation mixtures were brought to 0.033 M by the addition of carrier nucleosides. A variety of incubation conditions were used. In one experiment the inducer and radioactive precursors were introduced at the same time, and the induction was continued for 2 hr. In other experiments the tissue was preincubated with the radioactive precursors for 1 or 2 hr. before the addition of the inducer, and induction periods of 2, 3 and 4 hr. were used. In all experiments the incubation was carried out in the absence and presence of cycloheximide (2 μg /ml.), which should prevent translation and hence minimize the turnover of any messenger RNA involved in the induction (Kano-Sueoka & Spiegelman, 1962). After incubation the two samples of tissue were mixed and washed, and total nucleic acid was prepared. This was fractionated on an MAK column, and the fractions were counted simultaneously for ^3H and ^{14}C . Any differences in the $^{14}\text{C}/^3\text{H}$ ratio indicate differences in RNA synthesis specific for the incubation conditions used. For such differences to be meaningful the $^{14}\text{C}/^3\text{H}$ ratio must be constant for identical incubation conditions. The method of acid precipitation of the RNA and filtration on to Millipore membranes did not result in a constant $^{14}\text{C}/^3\text{H}$ ratio, even when one sample of tissue was incubated with both the ^3H - and ^{14}C -labelled precursors. This was due to the fact that the counting efficiency of ^{14}C was independent whereas ^3H efficiency was dependent on the amount of RNA precipitated (Fig. 1). Consequently the $^{14}\text{C}/^3\text{H}$ ratio varied with the concentration of the nucleic acid being eluted from the MAK column. This was overcome by counting aqueous samples of the RNA fractions in the toluene-dioxan-methanol scintillation medium (Fig. 1). This in turn involved the use of a volatile-salt gradient (NH₄HCO₃) to elute the column (Martin, 1963). The fractions were dried down by heat and the nucleic acid was dissolved in 0.5 ml. of water to which 15 ml. of the scintillation medium was added.

Purification of nitrate reductase. Considerable purification was achieved by gel electrophoresis. Homogenates, prepared as described for the measurement of nucleic acid and protein synthesis, were centrifuged for 30 min. at 100 000g. The supernatant was made 5% with respect to sucrose and 100 μl . was layered on to a 7.5 cm. $\times$ 0.63 cm. gel (7.5% acrylamide and 0.15% bisacrylamide in 0.05 M-tris-

* Abbreviation: MAK, methylated albumin on kieselguhr.

0.05M-glycine buffer, pH 8.9), which had been pre-run at 0°, 4mA/tube, at 250 v for 15 min. The electrophoresis was continued for 30–60 min. The gels were then removed from the tubes, and the position of the enzyme was located by incubation of the gel in a solution containing 1.5 ml. of 10 mM-potassium phosphate buffer, pH 7.5, 0.2 ml. of 0.1 M- NO_3^- , 0.5 ml. of 0.02% *N*-(1-naphthyl)ethylenediamine hydrochloride, 1 ml. of 0.5% sulphanilamide, pH 7.0, and 0.5 mg. of NADH for 20 min. at 30°. The gels were then transferred into 0.1 N-HCl, under which conditions the red azo-dye was formed from the nitrite (produced from the nitrate reductase activity), sulphanilamide and *N*-(1-naphthyl)ethylenediamine hydrochloride. Formation of the azo-dye was dependent on the presence of enzyme, nitrate and NADH. The gels were scanned at 550 m μ with a Joyce-Loebl Chromoscan within 30 min. of development of colour, since the dye slowly diffused out of the gel. After scanning, the total protein distribution on the gel was determined by staining in 0.01% Amido Black in methanol-acetic acid-water (5:1:5, by vol.) for 2–4 hr., followed by destaining in the methanol-acetic acid-water mixture for 2 or more days. The stained gels were scanned with a combination of 575 and 620 m μ filters. The distribution of [^{35}S]- or [^{14}C]-protein was determined by slicing the gels with a modified McIlwain chopper (Loening, 1967) and counting the dried 0.7 mm. sections on the Beckman Low Beta II instrument.

In some experiments the nitrate reductase was purified by calcium phosphate gel and $(\text{NH}_4)_2\text{SO}_4$ precipitation before electrophoresis. Calcium phosphate (10 mg.) was added to 4 ml. of the 100000g supernatant obtained from homo-

genizing 20 pairs of cotyledons in 6 ml. of medium. After centrifugation a further 13 mg. of calcium phosphate was added to the supernatant and the mixture was again centrifuged. The pellet was washed once with 1 ml. of the homogenization medium and then eluted with 1.3 ml. of 50 mM- K_2HPO_4 –10 mM-EDTA–5 mM-cysteine–4 mM- NO_3^- medium. Then $(\text{NH}_4)_2\text{SO}_4$ (300 mg.) was added slowly to 1 ml. of this solution. This mixture was left for 1 hr. at 0° and then centrifuged. The pellet was dissolved in 0.5 ml. of homogenization medium (plus 3 mM- NO_3^-) and used for assay of nitrate reductase activity, protein determination and electrophoresis.

RESULTS

The nitrate reductase activity was increased five- to ten-fold by incubation in nitrate. The use of ammonium nitrate as the inducer resulted in higher activities (Table 1). The incorporation of [^3H]uridine and of [^{14}C]leucine (or [^{35}S]sulphate) into RNA and protein during incubation of the cotyledons in nitrate was increased significantly over that of the chloride controls. This stimulation of synthesis did not appear to be directly associated with the induction of nitrate reductase activity, however, since incubation in ammonium nitrate, which induced greater activity, resulted in incorporation less than in the chloride controls (Table 1). The differences in total RNA and protein synthesis between nitrate and ammonium nitrate incubations were significant at the 5% and 2% levels respectively (Student's *t* test). The uptake of the precursors also varied, in a manner similar to the incorporation, with the different incubations (Table 1). However, the effect of the inducer anion on uptake was significantly smaller than the effect on incorporation, suggesting that the uptake may be a reflection of the synthetic requirements of the tissue rather than actively influencing the incorporation into macromolecules.

Fig. 1. Efficiency of counting [^3H]RNA (—) and [^{14}C]RNA (---) on Millipore membranes and in aqueous solution. ●, RNA precipitated without carrier and filtered on to membranes; □, RNA precipitated with 1 mg. of carrier RNA and filtered on to membranes; △, RNA counted in aqueous solution (see the Materials and Methods section).

Table 1. *Effect of induction on total RNA and protein synthesis*

Nitrate reductase activity was induced by KNO_3 and NH_4NO_3 , with KCl as the control, as described in the Materials and Methods section. Total RNA and total protein syntheses were corrected to counts/min. incorporated per mg. of RNA or mg. of protein respectively. Uptake of leucine (or sulphate) was measured as the ^{14}C (or ^{35}S) radioactivity in the initial homogenate. All values are expressed as percentages of the Cl^- control, $\pm$ s.e.m. of the numbers of experimental results (in parentheses).

Inducer	...	...	NO_3^-	NH_4NO_3
Nitrate reductase activity			710 $\pm$ 54 (10)	1100 $\pm$ 76 (8)
Total RNA synthesis			111 $\pm$ 6 (5)	83 $\pm$ 8 (3)
Total protein synthesis			119 $\pm$ 7 (10)	92 $\pm$ 7 (6)
Uptake of leucine (sulphate)			108 $\pm$ 5 (6)	98 $\pm$ 8 (4)

Table 2. *Effect of inhibitors on nitrate reductase induction and RNA and protein synthesis*

Nitrate reductase activity was induced with NO_3^- in the absence and presence of the inhibitors, as described in the Materials and Methods section. The results are expressed as percentage inhibitions caused by the inhibitor relative to the NO_3^- control. Minus values indicate stimulation.

Inhibitor	Concn. ($\mu\text{g./ml.}$)	Inhibition (%)		
		Nitrate reductase induction	RNA synthesis	Protein synthesis
Actinomycin D	10	25	68	27
	20	38	73	34
	40	61	84	38
5-Fluorouracil	325	-10	57	-14
Cycloheximide	0.2	20	2	5
	2.0	99	41	69
Puromycin	50	68	33	10
	100	87	38	7
	200	95	56	30
Chloramphenicol	1000	10	58	14
	2000	53	80	59

Fig. 2. Fractionation of nucleic acids from radish cotyledons and from 5 mm. root tips of soya-bean seedlings: MAK column chromatography of cotyledon (a) and root-tip (b) nucleic acids; gel electrophoresis of cotyledon (c) and root-tip (d) nucleic acids. The RNA in (a) was prepared from cotyledons labelled with $[^{32}\text{P}]$ phosphate for 2 hr. —, E_{260} ; ----, radioactivity. Abbreviations: s-RNA, soluble RNA; lr-RNA, light ribosomal RNA; hr-RNA, heavy ribosomal RNA.

The induction of nitrate reductase activity was inhibited by actinomycin D, cycloheximide, puromycin and chloramphenicol (Table 2). In all cases RNA and protein syntheses were both inhibited

independent of the specificity of the inhibitor. The relatively slight inhibition of amino acid incorporation by puromycin, under conditions where induction was inhibited by 90%, was presumably

Table 3. *Base compositions of tissue RNA and newly synthesized [³²P]RNA*

Base compositions are expressed as μ moles of nucleotide/100 μ moles. The composition of total RNA was determined by measuring E_{260} and of newly synthesized [³²P]RNA by the distribution of ³²P radioactivity (Ingle *et al.* 1965).

	Composition (%)				GMP AMP ratio
	CMP	AMP	GMP	UMP	
Total RNA	22.9	23.8	29.9	23.4	1.26
Newly synthesized [³² P]RNA					
[³² P]Phosphate present for 0.5 hr. at different times during a 4 hr. incubation					
0-0.5 hr.	21.3	31.8	23.8	23.0	0.75
0.5-1.0 hr.	21.4	31.4	23.9	23.2	0.76
1.5-2.0 hr.	21.1	33.3	23.1	22.5	0.69
3.5-4.0 hr.	22.3	32.2	23.5	22.0	0.73
[³² P]Phosphate present continuously 0-6 hr.	20.4	27.0	27.5	25.1	1.02

due to incorporation into incomplete, inactive, peptide chains (Nathans, 1964), which were assayed as protein under the experimental conditions. The induction of nitrate reductase was more sensitive than total protein synthesis to cycloheximide, but chloramphenicol, which has been reported preferentially to inhibit induced protein synthesis in bacteria, showed no such effect (Sypherd, Strauss & Treffers, 1962). 5-Fluorouracil inhibited RNA synthesis by 57%, but had no inhibitory effect on the induction of activity or on protein synthesis; in fact small but significant stimulations were observed.

RNA synthesis during induction. Fractionation on MAK columns of total nucleic acid prepared from the green radish cotyledons produced an elution pattern different from that obtained with RNA from tissues such as etiolated hypocotyl or root tips (Figs. 2*a* and 2*b*). Four low-molecular-weight components were present in the cotyledon RNA compared with the two components from root-tip RNA. Three or four high-molecular-weight RNA peaks were partially separated in the ribosomal RNA region, the first of these peaks being the largest. Much better resolution of the RNA components was achieved by polyacrylamide-gel electrophoresis (Figs. 2*c* and 2*d*). The cotyledons contained three additional RNA peaks (23s, 16s and 13s), which have been shown to be present in all green tissues studied, and which appear to be present exclusively in the chloroplast (Loening & Ingle, 1967).

Despite these differences in total RNA components between green cotyledons and non-green tissues, a type of RNA very similar to the DNA-like RNA (D-RNA) studied with hypocotyl and root-tip tissues (Ingle *et al.* 1965; Ingle & Key, 1965) was

Fig. 3. Inhibition of nucleic acid synthesis by 5-fluorouracil and actinomycin D. Cotyledons were preincubated for 1.5 hr. in the absence (*a*) and presence of 325 μ g. of 5-fluorouracil/ml. (*b*) and 20 μ g. of actinomycin D/ml. (*c*). [³²P]Phosphate (1 mc) was added and the incubation was continued for 4 hr. —, E_{260} ; ----, radioactivity.

synthesized in the cotyledons. After short incubations with [³²P]phosphate most of the newly synthesized RNA was eluted from the MAK column after the ribosomal RNA (Fig. 2*a*), a characteristic property of the D-RNA. The composition of the newly synthesized RNA was very different from that of total RNA, having high AMP content with

the correspondingly low GMP/AMP ratio approx. 0.73 (Table 3). The composition changed as a period of incubation in [^{32}P]phosphate increased,

Table 4. *Inhibition of the synthesis of nucleic acid components*

Nucleic acid synthesis was measured by the incorporation of [^{32}P]phosphate. Actinomycin and 5-fluorouracil were used at 20 $\mu\text{g./ml.}$ and 325 $\mu\text{g./ml.}$ respectively. Total nucleic acids were fractionated on MAK columns (Fig. 3), and the radioactivity of each component was summed. Approximate correction was made for the contamination of D-RNA by ribosomal RNA (Ingle & Key, 1965). The results are expressed as percentage inhibitions caused by the inhibitor relative to the control.

	Inhibition (%)	
	Actinomycin D	5-Fluorouracil
Total nucleic acid	91	43
Soluble RNA	83	28
DNA	83	60
Ribosomal RNA 1	99	93
Ribosomal RNA 2	98	87
D-RNA	94	18

approaching that of the total RNA of the tissue, the GMP/AMP ratio of which was 1.26. This change in the composition of [^{32}P]RNA with increasing time of incubation was due to turnover, rather than to a decreased rate of synthesis of the D-RNA relative to ribosomal RNA, since a 30 min. incubation with [^{32}P]phosphate at any time during a 4 hr. experimental period resulted in total RNA with very similar composition (Table 3).

Treatment of the cotyledons with actinomycin D resulted in approx. 90% inhibition of the synthesis of all the nucleic acid components (Figs. 3a and 3c). 5-Fluorouracil also inhibited ribosomal RNA synthesis by 90%, inhibiting the synthesis of soluble RNA by 30% and D-RNA by less than 20% (Fig. 3b and Table 4). Since it is known that much of the incorporation into soluble RNA is due to the addition of terminal nucleotides rather than complete molecular synthesis (Ingle *et al.* 1965), these data indicate that the induction of nitrate reductase activity, which is inhibited by actinomycin D but not by 5-fluorouracil, requires the continual synthesis of D-RNA, and perhaps soluble RNA, but not ribosomal RNA.

Fig. 4. Double labelling of RNA during the induction of nitrate reductase. Cotyledons were incubated in the absence (a) and presence of 2 $\mu\text{g.}$ of cycloheximide/ml. (b) with ^3H - and ^{14}C -labelled precursors of nucleic acids (see the Materials and Methods section). The E_{260} (—) and radioactivity (---) profiles are those from Expt. 1. ●, $^{14}\text{C}/^3\text{H}$ ratio of each fraction from the induced/non-induced tissue mixture; ○, $^{14}\text{C}/^3\text{H}$ ratio of each fraction from the non-induced/non-induced tissue mixture. Expt. 1: cotyledons were incubated for 1 hr. with radioactive precursors before the addition of NO_3^- or Cl^- , and cycloheximide, for a further 3 hr. Expt. 2: cotyledons were preincubated with precursors for 2 hr. before a 2 hr. induction period.

Fig. 5. Fractionation of soluble protein from radish cotyledons. Cotyledons were incubated with: (a) KCl; (b) NH_4Cl ; (c) KNO_3 ; (d) NH_4NO_3 ; the soluble protein was fractionated by electrophoresis for 1 hr. Total protein distribution (—) and nitrate reductase activity (----) were determined as described in the Materials and Methods section. The protein distribution between regions I, II, III and IV on the gel (d) was calculated as a percentage of total soluble protein applied to the gel, corrected for the fact that 20% of the protein had moved off the end of the gel under these conditions.

The doubly-labelled isotope technique was used to look for any relatively small changes in RNA synthesis, particularly in D-RNA synthesis, which may be associated with the induction of nitrate reductase. Cotyledons were induced in the absence (Fig. 4a) and presence (Fig. 4b) of cycloheximide, which should prevent translation and hence minimize turnover of messenger RNA. The cycloheximide affected RNA synthesis, preferentially preventing the accumulation of radioactivity in ribosomal RNA with essentially no effect on D-RNA synthesis (Key, 1966). Though the method of counting aqueous samples in the toluene-dioxan-methanol scintillation medium greatly improved the constancy of the $^{14}\text{C}/^3\text{H}$ ratio, fluctuations still occurred, particularly before elution of the ribosomal RNA peaks. With the range of incubation and induction conditions used (see Fig. 4), no significant differences in the $^{14}\text{C}/^3\text{H}$ ratio of the different types of RNA were observed between the induced/control and the control/control systems. The small stimulation of RNA synthesis resulting from nitrate incubation (Table 1) could thus be due to the equal stimulation of all types of RNA.

Protein synthesis during induction. The assay for nitrate reductase on the gel after electrophoresis of

the 100 000g supernatant showed a bimodal peak of activity. Induction by nitrate, ammonia and ammonium nitrate resulted in similar peaks of activity (Fig. 5). The distribution of total protein, as determined by staining with Amido Black, showed no significant changes with the different induction conditions. Essentially all of the protein moved towards the anode, and after 60 min. of electrophoresis 20% of the applied soluble protein had moved off the end of the gel. Of the protein remaining on the gel about 70% moved as a dense band (Fig. 5). Less than 4% of the stainable protein was in the region of nitrate reductase activity. This would represent 3% of the soluble protein applied to the gel or only 1.5% of the protein of the cotyledons, since the 100 000g supernatant contained less than 50% of the total protein. The recovery of enzyme activity on the gel was 70–90% of that initially applied, when corrections were made for the effects of polyacrylamide and pH on the assay.

Synthesis of new protein during the induction period was determined by the incorporation of radioactive precursors, [^{35}S]sulphate or [^{14}C]leucine. Similar results were obtained with both precursors. All of the proteins fractionated by electrophoresis incorporated label. The induction of nitrate

reductase did not significantly stimulate protein synthesis in the region containing the enzyme activity when compared with the non-induced chloride control (Fig. 6a). The distribution of the

newly synthesized protein was such that about 11% was present in the region of nitrate reductase activity.

Purification of nitrate reductase by calcium phosphate gel and ammonium sulphate precipitation resulted in a 30-fold purification of the enzyme (Table 5), though the recovery of enzyme activity was less than 5%. This purification did not result in a significant increase in the radioactivity associated with the nitrate reductase (Table 5). Electrophoresis of this purified nitrate reductase fraction resulted in a fairly narrow band of protein and radioactivity around the region active in nitrate reduction, but no differences were observed between the induced and non-induced systems (Fig. 6b).

DISCUSSION

The inhibitor data indicate that the induction of nitrate reductase activity requires the synthesis of RNA and protein. RNA metabolism in the radish cotyledon is more complex than in the non-green or etiolated tissues more commonly studied. Two additional low-molecular-weight and three additional high-molecular-weight types of RNA are present in the green tissues, and appear to be restricted to the chloroplast (Loening & Ingle, 1967; Dyer, 1967). However, during incubations of 2 hr. or less much of the newly synthesized RNA has characteristics similar to those of the D-RNA described in hypocotyl and root tips (Ingle *et al.* 1965; Ingle & Key, 1965). This RNA, having a composition with high AMP content and being unstable relative to the ribosomal components, has been compared with messenger RNA (Ingle *et al.* 1965). It appears that the synthesis of D-RNA only is required for induction, since 5-fluorouracil inhibits ribosomal RNA synthesis almost com-

Fig. 6. Protein synthesis during induction. Cotyledons were incubated in KCl or NH_4NO_3 and 2 mc of $[^{35}\text{S}]$ sulphate for 4 hr. Samples of the soluble protein from the induced (Δ) and non-induced ($\bullet$) tissue were fractionated by electrophoresis for 45 min. (a). The distribution of newly synthesized protein was calculated as a percentage of the radioactivity on the gel. Nitrate reductase was purified from the remaining material (Table 5) and subjected to electrophoresis (b) for 30 min. —, Protein; ----, nitrate reductase activity.

Table 5. *Purification of nitrate reductase before electrophoresis*

Nitrate reductase was induced with NH_4NO_3 , with KCl as the control, for 4 hr. in the presence of 1 mc of $[^{35}\text{S}]$ -sulphate and was purified as described in the Materials and Methods section. Nitrate reductase activity and μg . of protein were determined on 0.1 ml. portions of the various fractions.

Stage of purification	Nitrate reductase activity (milliunit)	Protein (μg .)	Nitrate reductase sp. activity (milliunits/mg. of protein)	Radioactivity incorporated (counts/min./ μg . of protein)
NH_4NO_3 -induced tissue				
100 000g supernatant	0.96	110	8.7	240
Calcium phosphate gel	0.50	8.5	59.0	251
$(\text{NH}_4)_2\text{SO}_4$ fraction	0.27	1.0	270	223
Cl^- control tissue				
100 000g supernatant	0.07	100	0.7	277
$(\text{NH}_4)_2\text{SO}_4$ fraction	0.02	1.0	20.0	260

pletely and the synthesis of soluble RNA by more than 30% without affecting the induction or total protein synthesis, whereas actinomycin D, which inhibits the synthesis of D-RNA as well as soluble RNA and ribosomal RNA, inhibits the induction of nitrate reductase activity. A similar but more clear-cut example of the requirement for D-RNA synthesis only has been shown for the growth of excised hypocotyl tissue (Key & Ingle, 1964). It is, however, difficult to inhibit the induction of nitrate reductase completely; 40% induction still occurs when only 15% of total RNA synthesis remains.

The small stimulation of total RNA synthesis by nitrate (Ingle, Beevers & Hageman, 1963) could be due to the equal stimulation of synthesis of all types of RNA. The induction of nitrate reductase does not stimulate total D-RNA synthesis relative to soluble RNA or ribosomal RNA synthesis, nor does it alter the distribution of D-RNA molecules to any extent, as analysed by MAK column chromatography of doubly-labelled RNA. Studies with *Escherichia coli* have shown that messenger RNA synthesis specific for an induced enzyme system represents only 1–2% of the total pulse-labelled RNA (Attardi, Naono, Rouviere, Jacob & Gros, 1963), which is a change too small to be detected by these methods.

About 100-fold purification of nitrate reductase was achieved by using electrophoresis. Only 1.5% of the total protein is associated with the region of nitrate reductase activity after electrophoresis. The amount of protein in this region is not increased under conditions by which the nitrate reductase activity has been increased 20-fold. In fact, if only 5% of the protein in the nitrate reductase activity peak were nitrate reductase, then a 20-fold increase in this would double the amount of protein in this region relative to the other protein components, and this would be easily detectable on the gels. The results therefore indicate that, if induction does involve new synthesis of protein, the amount of synthesis under the conditions used is 0.01%, or less, of the total protein of the cotyledon. On the basis of [³⁵S]sulphate or [¹⁴C]-leucine incorporation, the nitrate reductase region on the gel contains about 10% of the newly synthesized soluble protein, which would be 5% of the total protein synthesis. Again a 20-fold stimulation of as little as 2% of this would be easily detected by radioactive distribution on the gel. This indicates that synthesis of nitrate reductase during induction must be less than 0.1% of the total protein synthesis occurring under these particular conditions. As discussed previously, the induction of nitrate reductase is maximal at a stage of development when the cotyledon might be expected to have low general metabolic activity, since it is not growing in terms of cell size or number

and there is no net protein synthesis (Ingle *et al.* 1966). However, as shown in Fig. 6, radioactivity is incorporated into all the protein fractions. In the two reported cases of incorporation of radioactive precursor into a specific protein in plant tissues, induced thymidine kinase in microspores represented about 40% of the newly synthesized soluble protein (Hotta & Stern, 1963), and gibberellic acid-induced α -amylase in aleurone tissue contained 12% of the total incorporated radioactivity (Varner & Ram Chandra, 1964). These results presumably reflect in part the limited synthetic capacity of these highly specialized cells, and therefore cannot be directly compared with the radish cotyledons, where less than 0.1% of the newly synthesized protein could be nitrate reductase.

The induction of nitrate reductase in the radish cotyledon involves only a very small percentage of the protein, either on the basis of total protein present or of the protein synthesized during the induction period. It is therefore possible that the requirement of protein synthesis for induction, as determined by inhibitor studies, is not directly for nitrate reductase protein, but may involve the synthesis of an effector necessary for nitrate reductase activity.

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