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Purine Metabolism in Germinating Wheat Embryos

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1. Both the acid-soluble fraction and the nucleic acid fraction of wheat embryos were extensively labelled after incubation for 6hr. in the presence of [8-^{14}C]adenine. Subsequent incubation in the absence of labelled adenine resulted in no loss of radioactivity to the medium during a 48hr. period. Radioautography indicated that during this period there was a continuous increase in the radioactivity present in the acid-insoluble fractions of the root and leaf tissues relative to that present in the coleorhiza and coleoptile. 2. During incubation at 25° there was a 26-fold increase in the activity of 3′-nucleotidase between 4hr. and 24hr.; the activities of enzymes hydrolysing AMP and IMP increased to a smaller extent. The activities of adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase increased three- to five-fold during incubation at 25° for 24hr. 3. Adenosine kinase, inosine phosphorylase and 5-phosphoribosyl pyrophosphate synthetase activities were high in extracts from dry embryos and did not increase during 48hr. at 25°. 4. The increase in 3′-nucleotidase activity was prevented by cycloheximide, cryptopleurine or incubation at 4°, but not by actinomycin D; these treatments did not depress the activity of the other enzymes measured. 5. The results are discussed in relation to RNA translocation within the wheat embryo during germination.

Various workers have shown that RNA is translocated within plant seeds during the first few days after germination (Oota, Fujii & Osawa, 1953; Osawa & Oota, 1954; Oota & Takata, 1959; Barker & Douglas, 1960; Cherry & Hageman, 1961; however, see Ingle & Hageman, 1965). Both translocation of intact RNA molecules and translocation after degradation to acid-soluble material have been suggested by these authors. Recent studies have indicated that RNA disappears from the coleorhiza surrounding the root tissue during the first 2days after imbibition of wheat embryos at 25° (Price, 1969). Ribonuclease activity is high and increases during wheat-seed germination (Barker & Hollinshead, 1964; Vold & Sypherd, 1968), although gross measurements of activity are misleading as plant tissues contain several distinct nucleases that may differ in their distribution and function (Ingle & Hageman, 1965; Wilson, 1968*a*,*b*). In addition, 3′-nucleotidase activity has been shown to increase during wheat-embryo germination (Shuster & Gifford, 1962) and to be high in the coleorhiza and low in the root tissue (Price & Ey, 1969).

These results suggested that a period of extensive RNA and nucleotide catabolism occurs in the coleorhiza region shortly after imbibition of wheat embryos. This paper presents evidence that the purines released during degradation of RNA in the coleorhiza and coleoptile are reutilized by the embryo, probably by the neighbouring root and leaf tissues.

MATERIALS AND METHODS

Substrates and inhibitors. [8-^{14}C]Adenine, [8-^{14}C]hypoxanthine, [8-^{14}C]adenosine and [8-^{14}C]inosine were obtained from The Radiochemical Centre (Amersham, Bucks.).

Actinomycin D was obtained from Mann Research Laboratories (New York, N.Y., U.S.A.) and cycloheximide from Fluka A.-G. (Buchs SG, Switzerland). Cryptopleurine was kindly provided by Dr I. S. de la Lande.

The magnesium salt of PRPP* was obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.) and was converted into its sodium salt before use as described by Tay, Lilley, Murray & Atkinson (1969).

Isolation of wheat embryos. Embryos were separated from wheat-seed fragments produced when South Australian-grown wheat was damaged before milling. The fragments were partially cleaned by sieving them, and then transferred to a sintered-glass funnel attached at the base to a compressed-air supply. The wheat-seed fragments were lifted by the air current and the lighter material (mainly aleurone fragments) was removed by blowing across the top of the funnel, the embryos being left behind. This fraction

* Abbreviation: PRPP, 5-phosphoribosyl pyrophosphate.

consisted of more than 90% (w/w) embryos, with endosperm fragments as the main contaminant; at no time were the seeds or seed fragments exposed to moisture or to extremes of temperature.

Incorporation of [8-^{14}C]*adenine into the acid-soluble and nucleic acid fractions of wheat embryos.* Seventy embryos were grown for 6hr. at 25° in 0·5ml. of 0·2mM-[8-^{14}C]-adenine (specific radioactivity 51·5μc/μmole), and then washed with several changes of water and with 0·05M- and 0·1M-sucrose; the washed embryos were placed on slopes of sterilized filter paper moistened with 0·1M-sucrose (13 embryos/paper) and left at 25°. There was no evidence of bacterial or fungal contamination at any stage during the incubation period. At intervals two embryos were transferred to 5% (w/v) glutaraldehyde in 0·1M-sodium phosphate buffer, pH 7·0 for subsequent radioautography (see below) and 11 embryos transferred to 95% (v/v) ethanol at 0°. The embryos were extracted again with cold ethanol and then with ice-cold $HClO_4$, which dissolved the acid-soluble fraction. The residual material was suspended in 0·5M-$HClO_4$ and extracted at 80° for 30min. to give the acid-insoluble fraction. Acid extracts were also made of the filter papers from each batch of embryos; the radioactivity in each fraction was measured by liquid-scintillation counting in Bray's (1960) solution.

The embryos fixed in glutaraldehyde were washed with several changes of 5% (w/v) glutaraldehyde, dehydrated and embedded in wax. Sections (10μm.) were cut and radioautographs prepared with Kodak stripping film AR10 (Kodak data books vol. 2, sheet no. SC10). The films were developed after 7 days and the distribution of grains over the sections was measured with a Barr and Stroud integrating microdensitometer at constant aperture. The data are presented as percentage distribution in the coleorhiza, root, coleoptile and leaf tissues; this method of presentation avoids slight differences in total radioactivity resulting from small variations in section thickness.

Germination of wheat embryos. Embryos were germinated at 25° or 4° on filter papers moistened with water placed in covered Petri dishes. No bacterial or fungal growth was apparent during the incubation periods.

Preparation of extracts. Embryos were homogenized for 10sec. at 4° in 10vol. of 0·05M-tris–chloride buffer, pH 7·8, with an Ultra-Turrax homogenizer. The supernatants after centrifugation at 100000g for 20min. were used for enzyme assays; proteins were determined by the method of Lowry, Bessey & Crawford (1949).

Assays of enzyme activity. Adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase were assayed as described by Murray, Wong & Friedrichs (1969) with 75μM-PRPP at 30°.

Adenosine kinase was assayed as described by Murray (1968); reaction mixtures contained (final concentrations) 50mM-potassium phosphate buffer, pH 7·0, 2·5mM-ATP, 2·5mM-$MgCl_2$ and 0·5mM-[8-^{14}C]adenosine (specific radioactivity 0·15μc/μmole).

Assay mixtures for phosphatase measurement contained (final concentrations) 70mM-tris–chloride buffer, pH 7·5, and 0·33mM-AMP, 0·33mM-IMP, 0·33mM-3′-AMP or 0·5mM-p-nitrophenol phosphate and extract in a final volume of 1·2ml. After incubation at 37° for 20min. reactions were stopped with 0·05ml. of conc. HCl, and 1ml. samples were assayed for inorganic phosphate (Weil-Malherbe & Green, 1951).

Inosine phosphorylase was measured in assays containing (final concentrations) 50mM-potassium phosphate buffer, pH 7·4, 0·33mM-[8-^{14}C]inosine (specific radioactivity 2μc/μmole) and extract in a final volume of 0·3ml. After incubation at 30° for 10min., reactions were stopped with 0·01ml. of conc. HCl and 0·05ml. samples were spotted on Whatman 3MM paper with hypoxanthine and inosine as internal markers. Chromatography in 5% (w/v) Na_2HPO_4 (saturated with 3-methylbutan-1-ol) separated hypoxanthine (R_F 0·58) and inosine (R_F 0·73); radioactivity was measured by liquid-scintillation counting.

PRPP synthetase was assayed as described by Murray & Wong (1967) with (final concentrations) 0·49mM-ATP, 1·0mM-ribose 5-phosphate and 12·5mM-$MgCl_2$.

RESULTS

Embryo material used. Fig. 1 shows a tracing made from a micrograph of a wheat-seed section indicating the major areas of the embryo and their relation to the rest of the seed. There are no mature vascular tissues present in the embryo during the first 24hr. of germination, although prominent pro-vascular strands lead from the scutellum and coleoptile to the interapical node. There are three major and two minor primary roots embedded in the coleorhiza

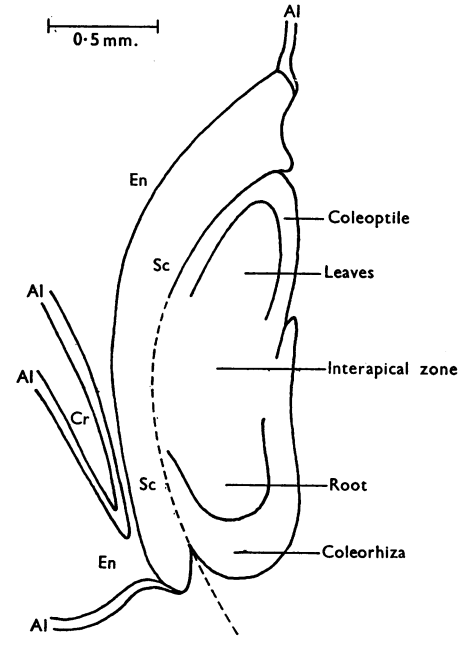

Fig. 1. Tracing of a photograph taken of a section of an unimbibed wheat seed. The section was prepared, stained and photographed as described by Price (1969). The broken line indicates the approximate line of weakness that enabled separation of the embryos from the rest of the seed to be performed. Al, aleurone layer; Cr, crease; En, endosperm; Sc, scutellum.

Table 1. *Incorporation of* [8-^{14}C]*adenine into the acid-soluble and acid-insoluble fractions of wheat embryos*

Wheat embryos were pre-labelled with [8-^{14}C]adenine for 6hr., incubated for various times at 25° and extracted as described in the Materials and Methods section. Results are presented as $10^{-3}\times$ c.p.m./embryo or as $10^{-3}\times$ total c.p.m. extracted from the paper on which each 13 embryos were incubated. The values have been corrected for a background count of 35c.p.m.

	Radioactivity			
Fraction Time of incubation (hr.) ...	4	12	24	48
Paper	0·06	0·04	0·03	—
Acid-soluble	13·8	14·9	10·1	5·4
Acid-insoluble	21·4	31·1	21·4	28·8
Total (embryo)	35·2	46·0	31·5	34·2
Acid-insoluble/acid-soluble radioactivity ratio	1·6	2·1	2·1	5·3

Table 2. *Incorporation of* [8-^{14}C]*adenine into the acid-insoluble fractions of wheat embryos*

Wheat embryos were pre-labelled with [8-^{14}C]adenine for 6hr. and incubated for various times at 25°, and radioautographs were prepared as described in the Materials and Methods section. Results are presented as means ± S.D. for the percentage distribution of grains in coleorhiza, root, coleoptile and leaf tissues.

Time (hr.)	Coleorhiza (C)	Root (R)	R/C	Coleoptile (Ct)	Leaf (L)	L/Ct
0	18·5±2·1	36·5±2·3	2·0	27·8±4·3	17·3±3·2	0·6
4	13·1±2·9	34·1±1·6	2·6	29·7±3·3	23·2±5·1	0·8
12	13·5±1·3	31·0±1·7	2·3	29·9±1·9	25·6±1·8	0·9
24	9·6±0·7	31·9±1·2	3·3	24·6±6·2	33·9±0·9	1·4
48	6·5±1·4	42·8±3·1	6·6	11·7±2·3	39·0±5·5	3·3

tissue. The embryos have a natural tendency to break away from the scutellum (see the broken line in Fig. 1) and it is isolated embryos of this type that were used in the experiments described in this paper.

Incorporation of [8-^{14}C]*adenine into the acid-soluble and acid-insoluble fractions of wheat embryos.* As previous studies (Price, 1969) had indicated that RNA was depleted in the coleorhiza during the first few days of germination, it was important to determine whether RNA-degradation products were released into the medium during this time or whether the RNA material was utilized by other regions of the embryo. To test this. embryos were pre-labelled with [8-^{14}C]adenine for 6hr. at 25°, washed and then transferred to filter papers and grown for a further 48hr. at 25° (for details see the Materials and Methods section).

Radioactivity present in the acid-soluble and acid-insoluble fractions and in the papers on which the embryos were grown at various time-intervals are shown in Table 1. The total radioactivity/embryo remained relatively constant, although there was a consistent increase with time in the proportion of the material present in the acid-insoluble (nucleic acid) fraction. At no time did the radioactivity in the filter papers on which the embryos were grown exceed 0·02% of the radioactivity of the embryos.

To determine the distribution of radioactivity in the acid-insoluble material between the different regions of the embryo, radioautography of embryo sections was carried out and the distribution of grains measured with a microdensitometer (see the Materials and Methods section); the results are shown in Table 2. The radioactivity associated with nucleic acids in the coleorhiza decreased continuously over the experimental period, whereas that in the coleoptile remained relatively constant for 24hr. and then decreased between 24hr. and 48hr. The total radioactivity in the root tissue remained relatively constant and that in the leaf tissue showed a continuous increase.

Activities of purine-metabolizing enzymes in wheat embryos. Utilization of nucleic acid-degradation products (nucleosides and bases) by the embryo would require the presence of enzymes able to 'salvage' these non-phosphorylated fragments. The activities of phosphatases, adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase, inosine phosphorylase, adenosine kinase and PRPP synthetase in extracts of wheat embryos at various times after incubation at 25° are summarized in Table 3. Hydrolysis of AMP and IMP increased over the 48hr. period, but hydrolysis of *p*-nitrophenyl phosphate also increased and dephosphorylation of these nucleotides may be due to

Table 3. *Activities of purine-metabolizing enzymes in extracts of wheat embryos during incubation at 25°*

Activities are expressed as nmoles/min./mg. of protein, and were assayed as described in the Materials and Methods section.

Time after imbibition (hr.) ... Activity measured	Activity: 0 (dry embryos)	2	4	6	16	24	40	48
AMP hydrolysis	0·7	0·6	0·6	2·1	2·8	2·6	5·0	4·7
IMP hydrolysis	0·9	0·0	0·1	1·1	2·1	2·1	5·0	3·0
3′-AMP hydrolysis	1·1	3·4	3·2	24·0	25·8	86·5	86·5	84·5
p-Nitrophenylphosphate hydrolysis	2·0	2·7	3·1	5·1	5·7	7·7	16·0	18·0
Adenine phosphoribosyltransferase	0·6	1·2	1·4	1·4	1·8	1·6	0·9	1·4
Hypoxanthine phosphoribosyltransferase	0·02	0·07	0·07	0·08	0·09	0·10	0·09	0·13
Inosine phosphorylase	2·6	1·2	3·2	3·0	3·2	3·2	3·3	3·3
PRPP synthetase	2·9	2·7	3·1	3·9	2·9	5·2	3·5	5·3
Adenosine kinase	3·1	5·3	6·4	5·2	4·7	5·4	4·0	5·3

Table 4. *Effect of protein-synthesis inhibitors and incubation at 4° on the activities of purine-metabolizing enzymes*

Extracts were made after incubation at 25° for 24hr. in the presence of 1mM-cycloheximide, 1mM-cryptopleurine or 0·05mM-actinomycin D or after incubation at 4° for 24hr. in the absence of inhibitor. Activities are expressed as nmoles/min./mg. of protein. —, Not determined.

Activity measured ... Treatment	Activity: Cycloheximide	Cryptopleurine	Actinomycin D	4°	25° (control)
AMP hydrolysis	1·6	1·3	6·9	4·8	2·6
IMP hydrolysis	1·9	0·5	4·6	0·0	2·1
3′-AMP hydrolysis	5·2	15·2	81·0	8·2	86·5
p-Nitrophenyl phosphate hydrolysis	5·9	5·6	7·2	4·7	7·7
Adenine phosphoribosyltransferase	2·3	2·3	2·0	1·8	1·6
Hypoxanthine phosphoribosyltransferase	0·06	0·12	0·10	0·07	0·10
Inosine phosphorylase	3·1	3·2	3·2	5·8	3·2
PRPP synthetase	—	—	—	5·2	5·2
Adenosine kinase	8·2	12·0	6·8	9·5	5·4

a non-specific phosphatase. However, the activity of 3′-nucleotidase increased 77-fold over the 48hr. incubation period; similar results were obtained by Shuster & Gifford (1962). Wilson (1968*a*) reported that 3′-nucleotidase activity was associated with a partially purified nuclease preparation from corn, but there is some evidence that hydrolysis of 3′-nucleotides is catalysed by a separate enzyme (Ingle & Hageman, 1965). The activity of adenine phosphoribosyltransferase increased twofold and that of hypoxanthine phosphoribosyltransferase sixfold over the 48hr. period; the true increases may in fact be greater, as the method of assay measures AMP and IMP formation, and hydrolysis of these nucleotides also increased during the experimental period. The activities of inosine phosphorylase, PRPP synthetase and adenosine kinase were high in extracts from dry seeds and did not increase during 48hr. of incubation at 25°; the activity of the latter enzyme is again a minimum one as the product of kinase action is also subject to hydrolysis.

Incubation at 25° in the presence of 1mM-cycloheximide, 1mM-cryptopleurine or at 4° in the absence of inhibitor suppressed the development of 3′-nucleotidase activity, indicating that the increase in activity observed resulted from protein synthesis (see Table 4). Cryptopleurine is a plant alkaloid that has been shown to inhibit protein synthesis in tumour cells (Donaldson, Atkinson & Murray, 1968) and in plant tissues (Polya, 1968). Subsequent incubation at 25° for 4hr. of embryos previously incubated for 24hr. at 4° resulted in no further increase in 3′-nucelotidase activity. Incubation with 0·05mM-actinomycin D did not inhibit the increase in 3′-nucleotidase activity (see Table 4). The activities of the other enzymes measured were not depressed by incubation with the protein-synthesis inhibitors or by incubation at 4° (Table 4). This

suggests that the increases in adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase activities observed at 25° (see Table 3) may represent enzyme activation rather than synthesis of new enzyme.

DISCUSSION

The results presented in this paper indicate that nucleic acid is depleted in the coleorhiza and coleoptile regions of wheat embryos during the first 2 days of germination, as suggested by Price (1969). This material is not released into the incubation medium but is redistributed within the embryo, particularly to the leaf tissues. The results are consistent with translocation at the macromolecular level, but the presence of nucleic acid- and nucleotide-hydrolysing enzymes and enzymes for reutilization of purines suggests that degradation to bases and nucleosides occurs first. If so, it would appear that the coleorhiza and coleoptile tissues of the wheat embryo act as storage tissues of RNA that is catabolized to non-phosphorylated fragments and reutilized by the leaf and root tissues during germination. Little movement of reserve materials from the endosperm or scutellum occurs during the first 24hr. of germination of wheat seeds, and the vascular system connecting the embryo with the scutellum is not complete until about 48hr. (C. E. Price and K. L. King, unpublished work). Thus the coleorhiza and coleoptile may play an important role in providing the embryonic roots and leaves with nutrients after imbibition. In view of the recent demonstrations of substituted adenines with cytokinin activity in plant transfer RNA (Bergquist & Matthews, 1962; Hall, Csonka, David & McLennan, 1967; Hall & Srivastava, 1967) and indications that cytokinins may act as free bases (Kende & Taveres, 1968), it is tempting to speculate that release of a specialized purine from RNA stored in coleorhiza and coleoptile tissues may initiate root and leaf growth.

It has been reported that messenger RNA is present in a masked form in dry wheat embryos and that this is activated after imbibition to support early protein synthesis (Marcus & Feeley, 1964, 1966; Marcus, Feeley & Volcani, 1966; Chen, Sarid & Katchalski, 1968). Chen *et al.* (1968) suggested that no new messenger RNA is synthesized during the first 24hr. of germination. These conclusions are entirely consistent with the present results, which indicate that actinomycin D had no inhibitory effect on the increase in 3′-nucleotidase activity during incubation at 25°.

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