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ACTIVITY OF ENZYMES INVOLVED IN PYRIMIDINE METABOLISM IN THE GERMINATING WHEAT GRAINS

BARBARA MAZUŚ and JERZY BUCHOWICZ

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

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Abstract—Activities of carbamoyl phosphate synthetase, aspartate carbamoyltransferase, dihydro-orotase, dihydro-pyrimidinase, uracil phosphoribosyltransferase, uridine phosphorylase, uridine kinase and acid phosphatase were measured in extracts from wheat grains germinated for periods ranging from 0 to 48 hr With the exception of uridine phosphorylase which appeared only 24 hr after initiation of germination, all these activities were detected in the dry grains Activities concerned with the synthesis of pyrimidine ring *de novo* dropped at the onset of germination (0 5–2 hr) and increased thereafter gradually Activities involved in the degradative pathway were maintained at a roughly constant level during the first 24 hr and rose considerably later on The results are discussed in relation to the sequence of initiation of RNA and protein synthesis during seed germination

INTRODUCTION

THE RELATIONSHIP of nucleic acid and protein synthesis in germinating seeds has received much attention recently The natural synchronization of biosynthetic processes, ordered with respect to the breaking of dormancy, offers a promising opportunity to establish a temporal pattern of reinitiation of nucleic acid and protein synthesis during germination. Although there is great activity in this field, it is difficult to conclude, as yet, whether the synthesis of RNA and protein are triggered simultaneously or in an order, and if so, in which order Many lines of evidence[1–6] indicate that it is the protein synthesis which starts immediately, prior to RNA, when appropriate changes in the environment allow seed to germinate Other evidence, however, supports the opposite view In particular, rapid incorporation of labelled orotate, uracil and uridine[7] indicates that RNA synthesis may be initiated immediately, being catalysed by enzyme systems apparently present in mature seeds Indeed, the activities of RNA polymerase[4] and some enzymes concerned with purine nucleotide synthesis[8] have been demonstrated in dormant seeds.

In attempts to extend these searches, studies on the activities of enzymes involved in the synthesis of pyrimidine moiety RNA precursors in dry and germinating wheat grains were undertaken

RESULTS

Activities of eight enzymes concerned with UMP synthesis and degradation were studied in dry and germinating (up to 48 hr) wheat grains Enzymes assayed were carbamoyl

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TABLE 1 ACTIVITY OF CARBAMOYL PHOSPHATE SYNTHETASE IN EXTRACTS FROM DRY AND GERMINATING WHEAT GRAINS

Germination time (hr)	Weight of the 100 grain sample fresh (g)	dry (g)	Concentration of protein in the extract (mg/ml)	Specific activity	Total activity
0 (dry grains)	4 7	4 2	9 0	0 08	0 07
0 5	5 6	4 2	8 5	0 05	0 04
1	6 3	4 2	8 4	0 04	0 03
2	6 3	4 1	8 4	0 05	0 04
4	6 4	4 1	8 3	0 17	0 14
6	6 6	4 1	8 1	0 28	0 23
12	6 8	4 1	7 6	0 38	0 29
24	7 5	3 9	7 8	0 75	0 59
48	9 6	3 8	9 1	0 71	0 65

Specific activity is expressed as nm's of the product formed per hr per mg of protein Total activity = specific activity × mg of protein extracted from one grain of the wheat The results represent the average of triplicate series Extraction and assay conditions are described in Experimental

phosphate synthetase (ATP carbamate phosphotransferase, E C. 2.7 2 5), aspartate carbamoyltransferase (carbamyl phosphate L-aspartate carbamoyltransferase, E.C. 2.1.3 2), dihydro-orotase (L-4,5-dihydro-orotate amidohydrolase, E C 3 5 2 3), dihydropyrimidinase (4,5-dihydropyrimidine amidohydrolase, E.C. 3 5 2 2), uracil phosphoribosyltransferase (UMP pyrophosphate phosphoribosyltransferase, E C 2 4 2 9), uridine phosphorylase (uridine orthophosphate ribosyltransferase, E C 2 4 2 3), uridine kinase (ATP uridine 5′-phosphotransferase, E C 2 7 1 48) and acid phosphatase (orthophosphoric monoester phosphohydrolase, E C 3 1 3 2) The results obtained for the individual activities are grouped in Tables 1–4, which indicate also the level of extractable protein found in the

TABLE 2 ACTIVITY OF ASPARTATE CARBAMOYLTRANSFERASE

Germination time (hr)	Concentration of protein in the extract (mg/ml)	Specific activity*	Total activity*
0 (dry grains)	8 9	74	66
0 5	8 7	40	35
1	8 7	40	35
2	8 7	36	31
4	9 0	36	32
6	9 1	52	47
12	8 7	120	104
24	8 5	150	128
48	10 0	222	222

* See Table 1 for the explanation

TABLE 3 ACTIVITY OF DIHYDRO-OROTASE AND DIHYDROPYRIMIDINASE

Germination time (hr)	Concentration of protein in the extract (mg/ml)	Dihydro-orotase Specific activity*	Dihydro-orotase Total activity*	Dihydropyrimidinase Specific activity*	Dihydropyrimidinase Total activity*
0 (dry grains)	8 0	0 14	0 11	0 04	0 03
0 5	7 4	0 07	0 05	0 05	0 04
1	7 3	0 07	0 05	0 05	0 04
2	7 3	0 11	0 08	0 04	0 03
4	7 6	0 15	0 11	0 04	0 03
6	7 6	0 17	0 13	0 05	0 04
12	7 0	0 31	0 22	0 06	0 04
24	7 4	0 37	0 27	0 13	0 10
48	8 6	0 46	0 40	0 33	0 28

* See Table 1 for explanation

corresponding enzyme extracts. Table 1 also includes data on changes in the fresh and dry weight of wheat grain sample during the period under investigation

With the exception of uridine phosphorylase, all activities tested were found to be invariably present in extracts from the dry grains. As germination proceeded, those activities changed in a pattern different for the individual enzymes However, for activities involved in the synthesis of the pyrimidine ring on the orotate pathway, i e carbamoyl phosphate synthetase (Table 1), aspartate carbamoyltransferase (Table 2) and dihydro-orotase (Table 3), there was a common tendency to drop at the onset of the germination period (0·5–2 hr) and to increase later on. This tendency is true for both specific and total activities, but the values calculated in the latter manner are more illustrative in this respect

The activity of uridine phosphorylase was not detectable until 24 hr after initiation of germination (Table 4) The activities of other enzymes concerned with catabolism, dihydropyrimidinase (Table 3) as well as uracil phosphoribosyltransferase and uridine kinase (Table 4), were high in extracts from dry seeds and did not change noticeably during the

TABLE 4 ACTIVITIES OF URACIL PHOSPHORIBOSYLTRANSFERASE, URIDINE PHOSPHORYLASE AND URIDINE KINASE

Germination time (hr)	Concentration of protein in the extract (mg/ml)	Uracil phospho-ribosyltransferase Specific activity*	Uracil phospho-ribosyltransferase Total activity*	Uridine phosphorylase Specific activity *	Uridine phosphorylase Total activity*	Uridine kinase Specific activity*	Uridine kinase Total activity*
0 (dry grains)	8 5	0 18	0 15	0 00	0 00	0 37	0 31
0 5	8 0	0 20	0 16	0 00	0 00	0 39	0 31
1	8 2	0 16	0 13	0 00	0 00	0 42	0 34
2	8 2	0 21	0 17	0 00	0 00	0 36	0 30
4	8 2	0 15	0 12	0 00	0 00	0 40	0 33
6	8 2	0 20	0 16	0 00	0 00	0 34	0 28
12	8 1	0 20	0 16	0 00	0 00	0 44	0 36
24	8 0	0 18	0 14	0 02	0 02	0 30	0 24
48	9 0	0 26	0 23	0 05	0 05	2 79	2 51

* See Table 1 for the explanation

first 24 hr There was, however, a significant increase in the activities of all degradative enzymes during the second day of germination This increase was particularly high (10-fold) in the case of uridine kinase

Phosphatase activity was not detectable when tested under conditions of the uracil phosphoribosyltransferase assay However, when the enzyme extracts were adjusted to pH 5 0, this activity, manifested by the conversion of UMP to uridine, appeared to be present in the wheat grains during the whole period

DISCUSSION

The results presented in this paper indicate that enzymes involved in the synthesis of UMP, on both orotate and degradative pathways, are present in extracts from dry wheat grains This observation, together with the data of Price and Murray[8] on the activities of PRPP synthetase, adenine and hypoxanthine phosphoribosyltransferases, inosine phosphorylase and adenosine kinase in unimbibed wheat embryos, and the report of Barker and Rieber[4] on the occurence of RNA polymerase activity in dormant pea seeds, strongly suggests that mature seeds may be endowed with a complete enzyme system potentially able to catalyse the synthesis of RNA from the simplest low-molecular-weight precursors Rapid incorporation of labelled RNA precursors *in vivo*[7] further indicates that these enzymes are actually operative from the onset of germination. Thus, two independent lines of evidence support the view that RNA synthesis may be initiated immediately after seed is allowed to germinate. The protein synthesis, on the other hand, seems to be triggered off considerably later. Neither of the investigated enzyme activities rose immediately after imbibition, at least 2 hr delay was observed A similar lag phase was observed in amino acid incorporation studied *in vivo*.[7,9]

One of the enzymes concerned with UMP synthesis, uridine kinase, has been previously[10,11] studied in relation to the germination process There is an apparent contradiction between our observation on the presence of uridine kinase activity in extracts from dry wheat grains and data of Schwarz and Fites[11] showing that this activity appears in the axes of peanut seeds only 24 hr after initiation of germination This discrepancy may result from differences in the experimental material used It is, however, probable that the charcoal treatment, employed in our procedure to minimize dilution effects, makes it possible to measure a very low level of the kinase activity Such an explanation may seem plausible in view of the low K_m value found for uridine kinase from corn seedlings [10] Thus our observations may be considered consistent with the data of Schwarz and Fites in that they show a rapid increase of the uridine kinase activity after 24 hr of germination Aspartate carbamoyltransferase has been recently isolated from dry wheat embryos.[12]

From the results presented, it may be concluded that mature wheat grain is endowed with enzyme systems allowing it to resume the synthesis of UMP, on both orotate and degradative pathways, immediately after initiation of germination

EXPERIMENTAL

Germination 100-grain samples of wheat, characterized in the previous paper,[7] were surface-sterilized with 2% $NaClO_4$ and germinated on sterile moistened filter paper in the dark at 21° for periods varying from 0 to 48 hr, as specified for each experiment

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Extraction of enzymes The whole sample of plant material was ground and homogenized with 10 ml of the appropriate buffer, correcting the volume used for water present in the imbibed grains The following buffers were used for the extraction of the several enzymes 0 05 M Tris-HCl, pH 8 0, for carbamoyl phosphate synthetase, 0 1 M Tris-HCl, pH 8 5, for aspartate carbamoyltransferase, 0 05 M potassium phosphate, pH 7 5, for dihydro-orotase and dihydropyrimidinase, and 0 1 M Tris-HCl, pH 8 0, for uracil phosphoribosyltransferase, uridine phosphorylase, uridine kinase and phosphatase Each buffer contained 0 1 mM 2-mercaptoethanol The homogenate was centrifuged at 15,000 *g* for 10 min The supernatant was used as the enzyme source The extract tested for the uracil phosphoribosyltransferase, uridine phosphorylase, uridine kinase and phosphatase activities was treated with 400 mg of activated charcoal and filtered prior to the use Extraction was performed at 2°

Enzyme assays Carbamoyl phosphate synthetase was assayed according to the method of O'Neal and Naylor,[13] using 50 μl portions of the extract as the enzyme source In this method $NaH^{14}CO_3$ (0 22 μc, specific radioactivity 7 5 μc/mole) serves as one of the substrates for the synthesis of [^{14}C]-carbamoyl phosphate, which is quantitatively converted into [^{14}C]-citrulline in the presence of excess of ornithine carbamoyltransferase and ornithine The radioactive citrulline formed on incubation (30 min at 37°) was identified and determined as described in the original method

Aspartate carbamoyltransferase was determined as described previously [14] The complete reaction mixture contained 100 μmoles of Tris-HCl buffer, pH 8 5, 0 1 μmole of 2-mercaptoethanol, 10 μmoles of carbamoyl phosphate, 20 μmoles of L-aspartate, enzyme (0 5 ml of the extract) and water to a final vol of 1 0 ml Incubation was at 37° for 30 min The reaction product, carbamoyl aspartate was isolated by column chromatography[15] and determined colorimetrically [16]

Dihydro-orotase was tested similarly as described previously,[17] using 0 11 μc of L-[^{14}C]-carbamoyl aspartic acid (specific radioactivity 0 11 μc/μmole), 100 μmoles of potassium phosphate–acetate buffer, pH 6 0, and 0 5 ml of the enzyme extract to prepare the reaction mixture (total vol , 1 ml) The reaction was stopped by addition of 5 ml of 0 3 M $HClO_4$ after 1 hr incubation at 37° Radioactive dihydro-orotate was isolated from the inactivated mixture by paper chromatography and quantitated by the radioactivity measurement The paper chromatography was carried out as described by Fink and Adams,[18] using *t*-BuOH–MeCOEt–H_2O–NH_4OH (4 3 2 1)

Dihydropyrimidinase assay contained 100 μmoles of diethanolamine buffer, pH 10 0, 1 4 μc of [^{14}C]-dihydrouracil (specific radioactivity 5 7 μc/μmole), 0 5 ml of the enzyme extract and water to a final vol of 1 0 ml Incubation was at 37° for 1 hr The product formed, [^{14}C]-carbamoyl-β-alanine, was isolated by the methods described previously[19] and assayed for radioactivity

Uracil phosphoribosyltransferase, uridine phosphorylase and uridine kinase activities were measured under conditions as described by Wasilewska and Reifer [20] The assay system for uracil phosphoribosyltransferase contained in a final vol of 1 0 ml Tris-HCl buffer, 100 μmoles (pH 7 4), 2-mercaptoethanol, 0 1 μmole, 2-[^{14}C]-uracil, 1 4 μc (specific radioactivity 5 7 μc/μmole), 5-phosphoribosyl pyrophosphate, 0 5 μmole, and enzyme (0 5 ml of the extract) To determine the uridine phosphorylase activity, the same reaction mixture was prepared, except that phosphoribosyl pyrophosphate was replaced by ribose-1-phosphate The reaction mixture for uridine kinase measurement contained 3 μmoles of ATP, 0 9 μc of 2-[^{14}C]-uridine (specific radioactivity 0 3 μc/mole) and Tris-HCl buffer, mercaptoethanol and enzyme in the same proportions as in the case of the uracil phosphoribosyltransferase assay After incubation at 37° for 30 min reactions were stopped with 1 ml of 0 6 M $HClO_4$ Radioactive UMP and uridine formed on incubation were isolated and determined quantitatively as described previously [21]

Phosphatase activity was tested with the use of 5′-UMP (1 0 μmole) as the enzyme substrate either under conditions of the uracil phosphoribosyltransferase assay or after acidification of the reaction mixture to pH 5 0 Uridine resulted from the dephosphorylation of UMP was separated by the method quoted above

General methods Protein concentration was determined according to the method of Lowry *et al* [22] Radioactivity was measured with a gas-flow Nuclear-Chicago GM counter, samples were plated on stainless-steel planchets, dried under an IR lamp and counted

Expression of results To avoid confusion between the activities of the enzyme preparations and the results of radioactivity determinations, the quantities of the products formed are expressed as *n*-moles, independently

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of the kind of the substrate (radioactive or non-radioactive) used Dilution effects were neglected when the molar quantity was calculated from the total radioactivity of the isolated product.

The results of enzyme activity measurements are presented in two ways (1) in terms of specific activity expressed as *n*-moles of the product formed per hr per mg of protein; (2) in terms of total activity, expressed as *n*-moles of the product formed per hr per wheat grain (i e per that quantity of protein which is extracted from one grain of the wheat)

To allow comparison of the results presented here with those calculated on other bases, the data on changes in fresh and dry weight of the standard 100-grain wheat sample during the period of investigation are given in Table 1

Reagents. [^{14}C]-Carbamoyl aspartate was prepared according to the method of Nyc and Mitchell.[23] [^{14}C]-Dihydrouracil was obtained by hydrogenation of 2-[^{14}C]-uracil [24] Ornithine carbamoyltransferase, purified 1000-fold from pea seedlings by the method of Kleczkowski and Cohen,[25] was a gift of Dr B Wielgat from our Institute Other reagents were of commercial sources

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