Phytochemistry, 1972, Vol. 11, pp. 3173 to 3179. Pergamon Press. Printed in England.

COMPOSITIONAL CHANGES DURING THE GERMINATION OF *CICER ARIETINUM**

S. AZHAR, A. K. SRIVASTAVA and C. R. KRISHNA MURTI
Division of Biochemistry, Central Drug Research Institute, Lucknow, India

(*Received* 3 *January* 1972. *Accepted* 23 *May* 1972)

Key Word Index—*Cicer arietinum*; Leguminosae; protein and polysaccharide degradation; soluble sugars; amino acids; phosphates; enzyme activities.

Abstract—Quantitative changes in the contents of protein, free amino acids, starch, polyfructosans, soluble sugars, phosphates and the activities of ten enzymes during 7 days of germination of *Cicer arietinum* (chick pea: Bengal gram) are reported.

THE PROTEIN reserves of *Cicer arietinum* are depleted during germination and the nitrogen content of the developing axis increases.[1] This is presumably the result of the release of amino nitrogen by the hydrolysis of reserve proteins and their translocation to the developing axis,[2] although no evidence has been adduced for the activation of any proteolytic system during the germination of this legume. The disappearance of over one-quarter of the reserve of RNA and DNA of *C. arietinum* during germination has, however, been shown to be accompanied by an increase in the activities of RNase and DNase.[3] Similarly, the decrease in the content of phosphatidyl choline of *C. arietinum* during germination is also marked by the concurrent appearance of a phospholipase D hardly detectable in the dormant seed.[4,5] The breakdown of polysaccharides and the utilization of the products during germination have not been investigated in this legume.

Although the mobilization of reserve food from storage tissue is well known to occur on seed germination, the regulatory mechanism has not been elucidated.There is overwhelming evidence for the view that the activation of enzymes occurring during germination arises out of *de novo* synthesis of proteins.[6,7]

Attempts to resolve the complexity of degradative changes during germination would be more meaningful if a balance sheet of such changes in terms of substrates and the relevant enzymes could be drawn up. The results of an investigation of the overall metabolism of proteins, polysaccharides and phosphates associated with the germination and early development of seedlings of *C. arietinum* are presented in this communication.

* Communication No. 1696 from the Central Drug Research Institute, Lucknow.

[1] N. A. RODINOVA, *Trudy, Glavnogo Botan. Sada. Akad. Nauk SSSR* **7**, 55 (1960); *Chem. Abs.* **55**, 21261d (1961).
[2] K. A. JADHAV and J. W. AIRAN, *J. Univ. Bombay Biol. Sci.* **29**, 11 (1960).
[3] S. M. HADI, *Ind. J. Biochem.* **3**, 203 (1966).
[4] R. T. TALWALKAR, N. K. GARG and C. R. KRISHNA MURTI, *Indian J. Exptl Biol.* **2**, 37 (1964).
[5] R. T. TALWALKAR, N. K. GARG and C. R. KRISHNA MURTI, *Indian J. Biochem.* **6**, 228 (1969).
[6] A. P. BROWN and J. L. WRAY, *Biochem. J.* **108**, 437 (1968).
[7] C. P. LONGO, *Plant Physiol.* **43**, 660 (1968).

RESULTS

Degradation of Polymer Constituents

The protein reserves of cotyledons were depleted by 50% in 7 days whereas within 5 days of growth the seedlings showed an increase of 30% in their net protein content (Table 1). Amino acids accumulated in the cotyledons as germination progressed and their concentration on the seventh day was 4-fold that in the dormant seed (Table 1).

TABLE 1. CHANGES IN PROTEIN, AMINO ACIDS, STARCH AND FRUCTOSANS CONTENTS DURING THE GERMINATION OF *C. arietinum* SEEDS

Germination (hr)	mg per gram dry wt Protein A	Protein B	Amino acids A	Amino acids B	Starch A	Fructosans A
0	263	(—)	5·9	(—)	530	11·0
24	234	(—)	15·3	(—)	500	10·8
48	214	100	16·2	85	475	9·0
72	205	117	17·1	105	450	8·5
96	183	130	19·0	109	425	7·7
120	163	137	20·4	115	375	6·6
144	145	145	23·0	120	350	5·5
168	130	155	24·0	120	275	4·6

A = Cotyledon; B = Seedling; (—) = Seedling not developed.
The starch and fructosan contents of seedlings were not determined.

Amino acid content of the seedlings was determined from 48th hr onwards and showed a 50% increase by the 6th day. The free amino acid content per unit mass was about 5-fold higher in the seedlings than in the cotyledons. Proteolytic activity of the cotyledons did not show any appreciable change as compared to that of the dormant seed, whereas the activity in the growing axis more than doubled (Table 2).

TABLE 2. CHANGES IN PROTEASE, α-GLUCOSIDASE, INDULASE AND INVERTASE ACTIVITIES DURING THE GERMINATION OF *C. arietinum* SEEDS

Germination (hr)	Enzymes units per mg protein Protease A	Protease B	α-Glucosidase A	α-Glucosidase B	Inulase A	Inulase B	Invertase A	Invertase B
0	36·8	(—)	0·30	(—)	20·0	(—)	32·0	(—)
24	37·4	(—)	0·23	(—)	22·0	(—)	33·6	(—)
48	40·0	160	0·17	0·83	86·0	2850	88·0	6100
72	38·2	210	0·30	0·94	50·0	1672	38·4	3013
96	39·2	240	0·32	1·12	55·0	400	25·8	853
120	42·0	260	0·37	1·30	60·0	383	52·0	1500
144	42·0	280	0·39	2·00	62·0	980	53·1	1700
168	42·4	360	0·41	1·63	70·0	1318	50·0	2123

A = Cotyledon; B = Seedling; (—) = Seedling not developed.

About 50% of the dry wt of seeds was constituted of starch and this was reduced to about 20% of dry wt by the 7th day in the cotyledons (Table 1). In contrast, the content of

polyfructosans was depleted about 47% in the cotyledons. The soluble sugars in the seeds were mainly hexoses (Fig. 3). On germination there was a slight rise in soluble sugar content in the cotyledons followed by a fall to the original level. In the seedlings, however, there was a sharp rise in the concentration of soluble sugar on the 3rd day of germination followed by a decrease to make the level slightly lower than that on the 2nd day. The pattern of changes in free hexose concentrations in the seedlings was similar to the changes in total soluble sugar. The reducing sugar content of cotyledons increased significantly during germination, reaching nearly a value three fold of that present in the dormant seed. The reducing sugar content of seedlings declined on the fourth day by about 15% and then began to rise to reach a level comparable to the content on the second day. Keto sugar concentration in the cotyledons increased by about 15% on the 2nd day and then remained unchanged throughout the rest of the period. In the seedlings, however, keto sugar concentration remained almost constant up to the sixth day and then dropped by nearly 50%. Pentoses increased uniformly in the cotyledons and seedlings, doubling their concentration over the period of analysis. Quantitatively the soluble sugar content of the seedlings was significantly higher than that of the cotyledons.

The concomitant changes in the activities of α-amylase are represented in Fig. 1 and the changes in activities of α-glucosidase, inulase and invertase are summarized in Table 2 α-Amylase activity in the cotyledons increased 6-fold during germination whereas the activity in seedlings showed only a 3-fold increase. α-Glocosidase activity measured with maltose as substrate showed a sharp fall in activity in the cotyledons up to 48 hr, but subsequently rose to a value higher than in the dormant seed. The corresponding activity in the seedlings showed a steady increase from the second day to the 6th day. The patterns of inulase and invertase activities were similar, with a 3-4-fold increase in cotyledons on the 2nd day, then a sharp fall and then a rise again. The activities in the seedlings also registered a sharp fall from the 2nd to the 4th day of germination. Phosphorylase activity (Fig. 2) showed significant increases both in the cotyledons and seedlings.

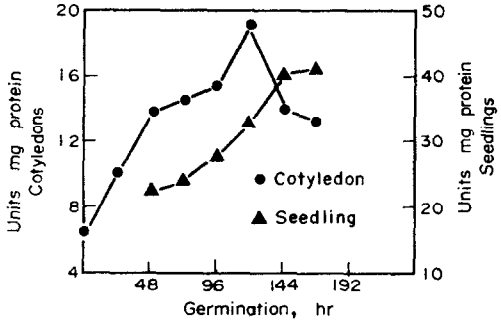

FIG. 1. CHANGES IN α-AMYLASE ACTIVITY DURING THE GERMINATION OF *C. arietinum*.
One unit of enzyme is the amount required to produce 0·1 mg reducing group (equivalent to maltose) in 10 min.

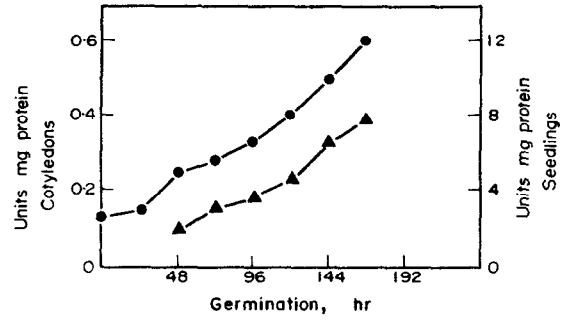

FIG. 2. CHANGES IN STARCH PHOSPHORYLASE ACTIVITY DURING THE GERMINATION OF *C. arietinum*.
One unit of enzyme is the amount required to produce 0·1 μmol P_i per 10 min.

Changes in Phosphate Concentration

The distribution pattern of total phosphorus and its resolution into acid soluble fractions and the further resolution of the acid soluble fraction into inorganic, labile and stable fractions is shown in Fig. 4. The phosphorus concentrations in fractions other than labile P

in cotyledons decreased by 30–50%. In the seedlings the total P, P_i, total acid soluble P and stable P all showed increases to varying extents. The stable P fraction of cotyledons decreased whereas that of the seedlings increased during the same period. The turnover of labile P was very rapid in the seedlings. Changes in the activities of phosphatases are shown in Fig. 5. Acid phosphatase activity was almost negligible in the dormant seed but rose about 15-fold in seven days in the cotyledons with a 3-fold rise in the seedlings between the 2nd and 7th days. Alkaline phosphatase activity also rose about 16 fold in cotyledons by the 4th day, after which it declined. In the seedlings, however, the activity increased by 2-fold from the 2nd day to the 7th day. The pattern of acid pyrophosphatase changes (unpublished data) in the cotyledons resembled the corresponding changes in acid phosphatase with a 7-fold increase in 7 days. The activity in the seedlings showed a 3-fold increase from the 2nd day to the 5th day and remained steady thereafter. The alkaline pyrophosphatase showed a 4-fold increase in activity in cotyledons initially and then dropped to its original level on the 5th day after which it increased again. In the seedlings this activity increased 7-fold from the 2nd to the 7th day.

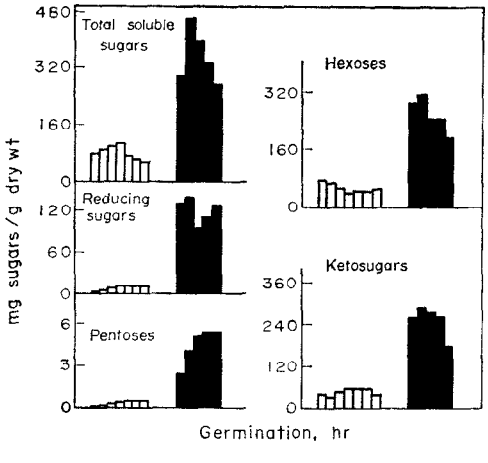

FIG. 3. CHANGES IN FREE SUGARS DURING THE GERMINATION OF *C. arietinum*.
Each bar represents change in 24 hr starting from 0 to 120 hr.

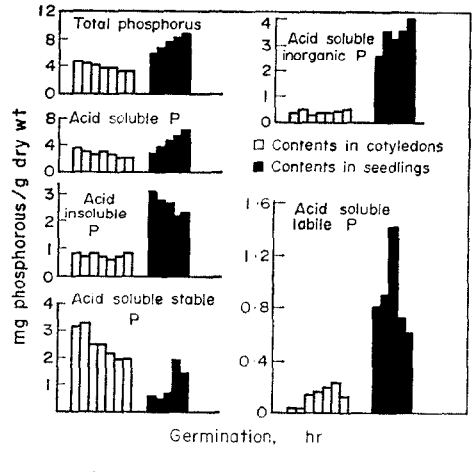

FIG. 4. CHANGES IN PHOSPHATE CONTENTS DURING THE GERMINATION OF *C. arietinum*.
Each bar represents change in 24 hr starting from 0 to 120 hr.

DISCUSSION

The depletion of protein from cotyledons and the accumulation of protein in the developing axis during germination of *C. arietinum* with a concomitant increase in free amino acids resemble the situation in peas.[8] The increase in activity of the proteolytic system in *C. arietinum* is consistent with the degradation of proteins. The net proteolytic activity of the growing tissue is several fold that of the cotyledons. The activity in the seedlings presumably arises out of synthesis of new proteins to sustain a growing tissue. Previous studies of Hadi and Krishna Murti[9] have revealed that the protein synthesizing ability of the

[8] J. D. HENSHALL and T. W. GOODWIN, *Photochem. Photobiol.* **3**, 243 (1964).
[9] S. M. HADI and C. R. KRISHNA MURTI, *Ind. J. Biochem.* **4**, 1 (1967).

growing tissue of this legume increases with the period of germination. The active turnover of proteins needed for the growth of tissue involved both synthesis and degradation. In the following paper, several germination inhibitors are shown to arrest most of the chemical activities reported in the present study. The enzymes involved in the utilization of polysaccharides and phosphate esters which increase in activity can also, therefore, be assumed to be arising by *de novo* synthesis from the amino acid pool enriched by the degradation of reserve proteins.

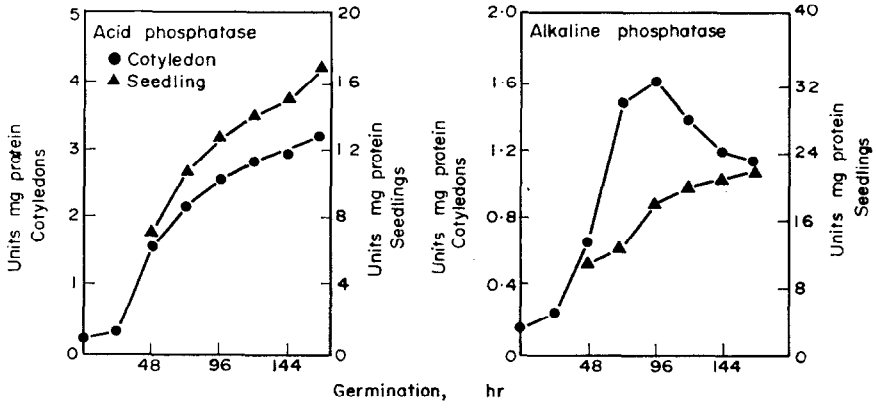

FIG. 5. CHANGES IN PHOSPHATASES ACTIVITIES DURING THE GERMINATION OF *C. arietinum*. One unit of enzyme is the amount required to produce 1 μmol P_i per 15 min.

Closely parallel to the degradation of reserve proteins during germination, starch and polyfructosans were also rapidly metabolized leading to the appearance in significant concentration of soluble and easily assimilable sugars. The activation of α-amylase and α-glucosidase during germination suggests that the degradation of starch is by hydrolytic cleavage. However, the concurrent activation of starch phosphorylase would imply that the phosphorolytic cleavage mechanism is also operating. The activation of alkaline and acid phosphatases, and pyrophosphatases lends support to this suggestion. Presumably sucrose arising from phosphorolytic cleavage in cotyledons is transported to seedlings where it is converted to glucose and fructose. Glucose-1-phosphate produced from starch by phosphorolytic cleavage can also enter the EMP pathway.

There is good evidence that organic phosphates are rapidly degraded by the appropriate phosphatases since the level of P_i in seedlings shows a significant increase. With the extensive breakdown of hexoses for energy production, it is reasonable to assume that the high level of P_i contributes to its fixation as high energy esters. Changes in the chemical constituents and activation of the appropriate enzymes in germinating *C. arietinum* are in agreement with the observations of Koller *et al.*[10] that the increase in respiratory rate and biosynthesis demands a concurrent degradation of reserve material. The mechanism by which these changes are regulated can be elucidated once the nature and characteristics of the enzymes that are synthesized *de novo* are known more fully.

[10] D. KOLLER, A. M. MAYER, A. POLJAKOFF-MAYBER and S. KLEIN, *Ann. Rev. Plant Physiol.* **13**, 437 (1962).

EXPERIMENTAL

Germination. *C. arietinum* seeds obtained from local grocery stores were soaked for 10 min in 0·1% calcium hypochlorite and washed thoroughly with distilled H_2O. They were then kept covered in distilled H_2O for 5 hr and germinated in the dark at 22 ± 1° on sheets of moist filter paper spread on acid-washed sand.

Processing of seedlings for analysis of proteins and amino acids. The seeds were separated into cotyledons and embryos after removing the seed coat. The component parts from 10 or more seeds as required were extracted for 5 min with 25 ml of boiling 60% EtOH. The tissue was then transferred to a pestle and mortar and ground to a fine slurry with 15 ml of boiling 60% EtOH. The slurry was centrifuged at 12 000 *g* for 10 min and the supernatant retained. The residue was extracted twice with 25 ml aliquots of the same solvent and the washings recovered by centrifugation were added to the original supernatant and used for the assay of amino acids. The residue was dried at 60° for 16 hr. 50 mg were triturated with 10 ml of 0·5 N NaOH and the suspension incubated at 37° for 4 hr. The supernatant recovered by centrifuging the suspension was used for the estimation of proteins.

Processing of seedlings for the analysis of starch, polyfructosans and sugars. 5 g samples of cotyledons or seedlings were ground to a paste with 20 ml of 80% EtOH and transferred to a round-bottomed flask. 10 ml of 80% EtOH were added and the suspension refluxed for 6 hr, cooled and filtered under suction. The alcoholic extract was concentrated under reduced pressure to 10 ml, treated with saturated lead acetate solution to precipitate pigments. The major part of lead in the supernatant was removed as lead oxalate and the extract then deionized by passing successively through beds of IR-120 and IR-450 prior to assay of soluble sugars. The residue was dried at 60° for 16 hr. Starch was isolated with 50% w/v perchloric acid,[11] and fructosans by the procedure of McRay and Slattery.[12]

Processing of seeds for phosphate analysis. 1 g of tissue was homogenized with 10 ml of ice cold 0·2 N perchloric acid and the homogenate centrifuged at 12 000 *g* for 10 min. The residue was re-extracted with perchloric acid and the washings were added to the original supernatant; the pooled extracts were used for the assay of acid soluble phosphate. The residue was digested with 2 ml conc. H_2SO_4 for 5 hr and the digest used for the assay of acid insoluble phosphate.

Enzyme assays. 10% w/v homogenates were made in 10 mM Tris-HCl buffer pH 7·0 and centrifuged at 800 *g* for 10 min and the supernatant used for the assay of different enzymes.

Enzymes were assayed as follows: *α-glucosidase* (α-D-glucoside glucohydrolase, E.C. 3.2.1.20), according to Laws and Moore;[13] *inulase* (β-1,2-fructan fructanohydrolase, E.C. 3.2.1.7) was assayed by a modification of the procedure described by Edelman and Jefford;[14] *invertase* (β-D-fructofuranoside fructohydrolase, E.C. 3.2.1.26), essentially according to Straus;[15] *α-amylase* (α-1,4-glucan-4-glucanohydrolase, E.C. 3.2.1.1), according to Bernfeld;[16] *phosphorylase* (α-1,4-glucan:orthophosphate glucosyl transferase, E.C. 2.4.1.1), according to Whelan[17] with the exception that P_i liberated in the backward direction was measured by the procedure outlined by Taussky and Shorr;[18] *proteolytic activity*, with casein as substrate, according to Young and Varner (1959);[19] *acid phosphatase* (orthophosphoric monoester phosphohydrolase E.C. 3.1.3.2), according to Appelman *et al.*;[20] *alkaline phosphatase* (orthophosphoric monoester phosphohydrolase, E.C. 3.1.3.1), according to Appaji Rao *et al.*;[21] *acid and alkaline pyrophosphates* (pyrophosphate phosphohydrolase, E.C. 3.6.1.1), according to Naganna *et al.*[22] and phosphorus liberated estimated by the method of Taussky and Shorr.[18]

Chemical estimations. Aminoacids were estimated by the ninhydrin method.[23] Protein in plant residue was determined by the microbiuret method of Itzhaki and Gill[24] and in homogenates by the colorimetric method of Lowry *et al.*[25] Total free sugars were assayed according to Lee and Montgomery,[26] total hexoses

[11] G. W. PUCHER, C. S. LEAVENWORTH and H. B. VICKERY, *Analyt. Chem.* **20,** 850 (1948).
[12] W. L. MCRAY and M. C. SLATTERY, *J. Biol. Chem.* **157,** 161 (1945).
[13] M. LAWS and J. H. MOORE, *Can. J. Biochem. & Physiol.* **41,** 2107 (1963).
[14] J. EDELMAN and T. G. JEFFORD, *Biochem. J.* **93,** 148 (1964).
[15] J. STRAUS, *Plant Physiol.* **67,** 342 (1962).
[16] P. BERNFELD, *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. I, p. 149, Academic Press, New York (1955).
[17] W. J. WHELAN, *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. I, p. 192, Academic Press, New York (1955).
[18] H. H. TAUSSKY and E. SHORR, *J. Biol. Chem.* **202,** 675 (1953).
[19] J. L. YOUNG and J. E. VARNER, *Arch. Biochem. Biophys.* **84,** 71 (1959).
[20] F. APPELMAN, R. WALTIAUX and C. DE DUVE, *Biochem. J.* **59,** 438 (1955).
[21] N. APPAJI RAO, H. R. CAMA, S. A. KUMAR and C. S. VAIDYANATHAN, *J. Biol. Chem.* **235,** 3352 (1960).
[22] B. NAGANNA, A. RAMAN, B. VENUGOPAL and C. E. SRIPATHI, *Biochem. J.* **60,** 215 (1955).
[23] E. W. YEMM and E. C. COCKING, *Analyst.* **80,** 209 (1955).
[24] R. F. ITZHAKI and D. M. GILL, *Anal. Biochem.* **9,** 401 (1964).
[25] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193,** 265 (1951).
[26] Y. C. LEE and R. MONTOGOMERY, *Arch. Biochem. Biophys.* **93,** 292 (1961).

by the anthrone method as standardized by Roe,[27] reducing sugars according to Park and Johnson,[28] or by the Nelson[29] and Somogyi[30] method, keto sugars according to Roe and Papadopoulos,[31] pentoses according to Tracey[32] and phosphorus according to Fiske and SubbaRow.[33]

Acknowledgement—The work was done during the tenure of Junior Research Fellowships awarded by the Council of Scientific and Industrial Research to S.A. and A.K.S.

[27] J. H. Roe, *J. Biol. Chem.* **212,** 335 (1955).
[28] J. T. Park and M. J. Johnson, *J. Biol. Chem.* **181,** 149 (1949).
[29] N. Nelson, *J. Biol. Chem.* **153,** 375 (1944).
[30] M. Somogyi, *J. Biol. Chem.* **160,** 62 (1945).
[31] J. H. Roe and N. M. Papadopoulos, *J. Biol. Chem.* **210,** 703 (1954).
[32] M. V. Tracey, *Biochem. J.* **47,** 433 (1950).
[33] C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.* **66,** 375 (1925).