Hexose-, Inositol-, and Nucleoside Phosphate Esters in Germinating Seeds of Crested Wheatgrass[1]

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Received June 13, 1966.

Summary. Net synthesis of phosphate esters was measured in crested wheatgrass seeds [*Agropyron desertorum* (Fisch.) Schult.] to learn what phosphorylating reactions occur in early stages of germination. Phosphate esters were separated on ion exchange columns and identified by cochromatography and chemical analyses. Synthesis of adenosine triphosphate and UDP-hexose was detected 6 hours after the beginning of water absorption. This synthesis occurred during the period of rapid increase in water potential. Synthesis of hexose phosphate and uridine triphosphate was detected 12 hours after the beginning of water absorption. The concentrations of these esters increased during 48 hours. Increases in concentration of inositol di-, tri-, and tetraphosphate suggested that hydrolysis of inositol hexaphosphate began after 12 hours.

Water in seeds plays a key role in the initiation of degradative and biosynthetic reactions that lead to the resumption of growth. The synthesis of nucleic acids (7), proteins (8), and cell walls occurs early in germination. These and other processes require phosphorylated substrates. The hydrolysis of inositol hexaphosphate provides phosphate needed for growth (1, 9, 10, 15). Our objective was to study, during the early stages of germination and in relation to seed water potential, the synthesis of phosphate esters and the hydrolysis of inositol hexaphosphate.

Materials and Methods

Crested wheatgrass seeds (4 g dry wt) were placed on moistened pyrex fiber at 23° for periods of 0, 6, 12, 24, 36, or 48 hours. Roots emerged after 48 hours.

Seed water potential was measured with a thermocouple psychrometer using the peltier effect (14) and the psychrometer designed by Campbell et al. (6). Sample temperature was maintained at 25 ± 0.001° in a water bath regulated by a Thermotrol[2] temperature controller (Hallikainen Instruments). Output of the chromel-constantan thermocouple was amplified and recorded using a microvolt indicating amplifier (Leeds and Northrup Company) and a servo-recorder (Heath Company).

Seed enzymes were denatured by using a low temperature procedure similar to the one described by Bieleski (4) and Bieleski and Young (5). After the desired period of water absorption, seeds were stored at −80° in 0.6N trichloroacetic acid dissolved in diethyl ether. Seeds were homogenized first in the trichloroacetic acid-ether solution at −40° to −80°. Homogenization was accomplished in a 250 ml Volu-mix container assembly (Lourdes Instrument Corporation). After centrifuging the homogenized material, approximately 5 μmoles of phosphate remained dissolved in the trichloroacetic acid-ether solution. This phosphate was recovered by washing the solution twice in a separatory funnel with 25 ml of water. When compared with this low temperature procedure of denaturing enzymes, boiling seeds in ethanol resulted in a 34% breakdown of ATP and a 51% breakdown of UTP. A small amount of inositol hexa-P was dephosphorylated in the boiling procedure, but the amounts of hexose-P, UDP-hexose, and other inositol-P esters appeared unaffected by method of denaturing enzymes.

After the denaturing procedure, the residue was homogenized 4 times at 0° to 4° in 50 ml of 0.3N aqueous trichloroacetic acid. Trichloroacetic acid was removed from the supernatant solution by extracting 4 times with 2 volumes of diethyl ether. The percentages of acid-soluble phosphate removed in 4 successive homogenizations were 59, 32, 7, and 2, respectively.

[1] Cooperative study of Crops Research Division, Agricultural Research Service, United States Department of Agriculture, and College of Agriculture, Washington State University. Scientific Paper 2790.

[2] Trade names are cited only for identification of equipment used.

Phosphate esters in the aqueous extract (ca. 250 ml) were adsorbed to the ion exchange column at 4° and the column was washed with 200 ml water. Phosphate esters were eluted at 23° with a gradient of formic acid and ammonium formate (fig 1). In preliminary trials, chromatography at 4° did not increase the amounts of phosphate esters isolated and gave a poorer separation than chromatography at room temperature.

Phosphate was determined by a modification of the Fiske-Subbarow method (2), hexose by a modification of the anthrone method (3), and nucleotides by UV absorbance. Phosphate esters were purified on a second ion exchange column before making hexose and UV measurements. In calculating the amounts of phosphate, hexose, or nucleoside in a compound, the amounts in successive fractions from the column were summed and baseline corrections were made. In measuring UV spectra, formic acid and ammonium formate in the effluent were removed under vacuum with the aid of a heat lamp. After removing formic acid and ammonium formate and hydrolyzing samples in 6N HCl at 100° for 24 hours, inositol was detected by using the yeast bioassay of Atkin et al., as reported by Snell (13).

Authentic compounds for cochromatography were purchased from Calbiochem. Enzymatic dephosphorylation products of inositol hexa-P were prepared essentially as described by Tomlinson and Ballou (15). Phytase was prepared from wheat meal by the method of Peers (11).

Results and Discussion

The identifications were made by using phosphate esters from seeds that had germinated 48 hours (fig 1). The molecular ratio of phosphate to hexose in hexose-P fractions was 0.9. No organic phosphate was detected in P_i fractions. The ratio of phosphate to nucleoside in ADP, ATP, and UTP fractions was 2.2, 2.8, and 3.1, respectively. The ratio of hexose: nucleoside: phosphate in UDP-hexose fractions was 1:1:2.3. When

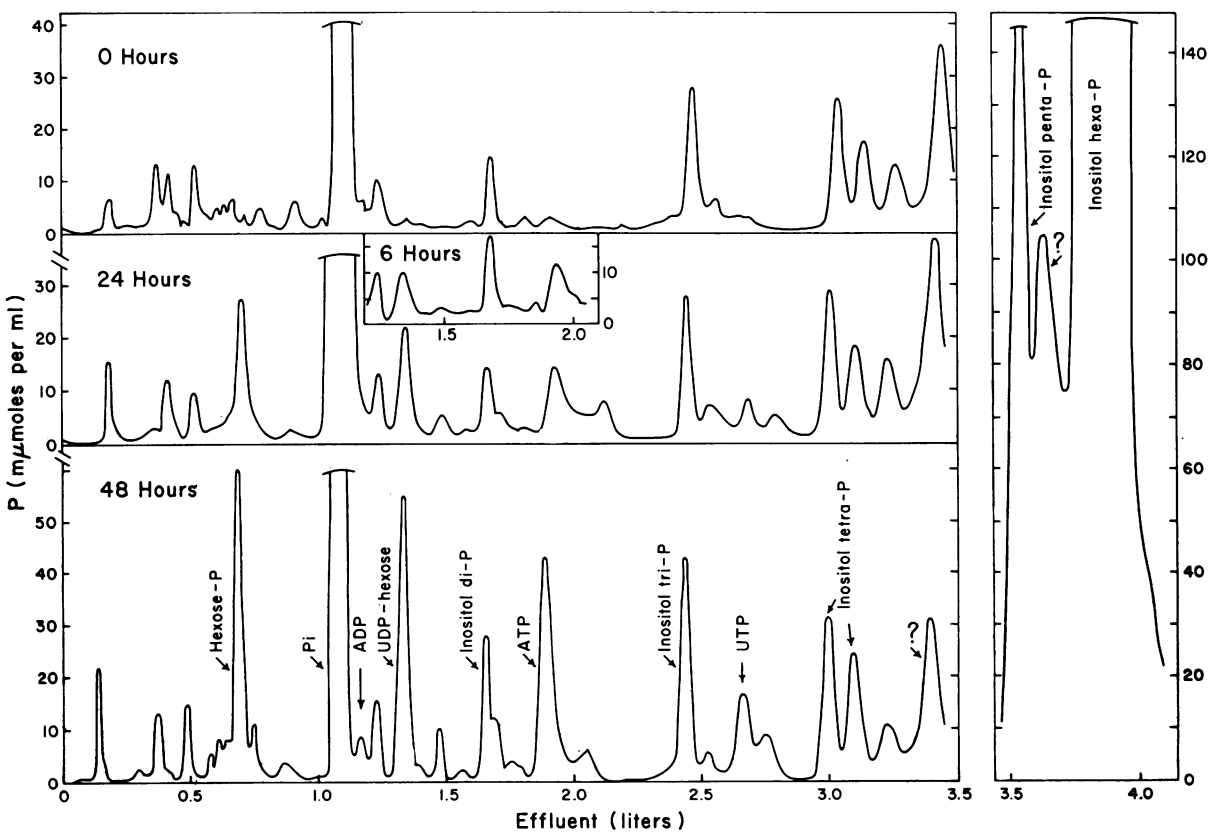
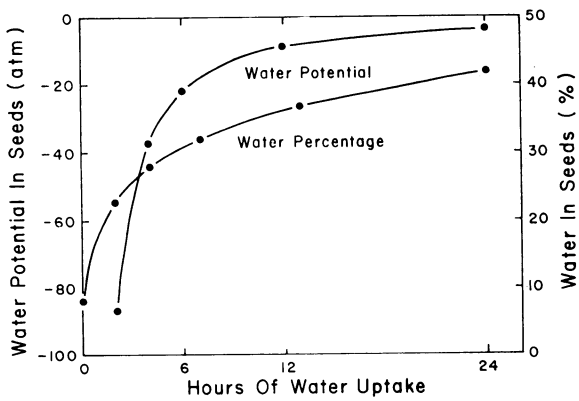


FIG. 1. Phosphate esters isolated from 4 g dry weight of seeds that had absorbed water for 0, 6, 24, or 48 hours. Inositol penta-P and inositol hexa-P did not measurably change during germination and are shown for the 48-hour treatment only. Net synthesis of UDP-hexose and ATP, detected 6 hours after the beginning of water uptake, is shown in the inset. Phosphate esters were eluted from the ion exchange column (Dowex-1 × 8, 140 to 325 wet mesh, 1.1 by 50 cm resin bed) with a gradient of formic acid and ammonium formate. Chambers 1 to 5 of the variable gradient device (12) initially contained formic acid concentrations of 0, 2.5, 4, 4, and 4 N and ammonium formate concentrations of 0, 0, 0.75, 2, and 2 M. Initial volume in each chamber was 1 liter. Fraction volume was 10 ml and flow rate was 50 ml per hour.

UDP-hexose was heated at 100° for 10 minutes in 1N HCl and the hydrolysis products separated, the ratio of UMP to P_i was 0.9. Nucleosides gave characteristic UV spectra. Authentic compounds cochromatographed on the ion exchange column with the identified phosphate esters.

Inositol-P esters from seeds cochromatographed on the column with enzymatically prepared inositol-P esters. On the basis of their position in the effluent, the esters were tentatively identified as inositol di-, tri-, tetra-, penta-, and hexa-P. Enzymatically prepared inositol mono-P was eluted immediately preceding hexose-P. Inositol was detected in all fractions of the effluent identified as inositol-P esters. The 2 inositol tetra-P esters are assumed to be isomers differing in the position of phosphate groups (15). Isomers that are not chromatographically separated may be present in other inositol-P fractions.

Seed water potential increased rapidly during the first 6 hours (fig 2). Synthesis of ATP and UDP-hexose occurred during this period of rapid increase in water potential (fig 1). Synthesis of hexose-P and UTP was detected after 12 hours of water absorption. The concentrations of these 4 esters increased during the 12 to 48 hour period. A number of other phosphate esters, present in trace amounts, were synthesized in early stages of germination. On the basis of availability of phosphorylated substrates, processes such as synthesis of nucleic acids, proteins, and cell walls could occur within 6 or 12 hours after the beginning of water absorption.



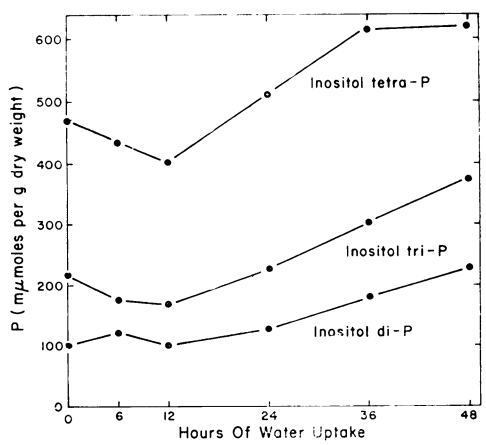


FIG. 2. (*top*) Increases in seed water percentage and water potential with time.

FIG. 3. (*bottom*) Changes in concentration of inositol di-, tri-, and tetra-P with itme.

The concentration of inositol hexa-P was 86 ± 7 μmoles P per g dry weight and did not measurably change during 48 hours. However, increases in concentration of inositol-P esters after 12 hours suggest that some dephosphorylation of inositol hexa-P was occurring (fig 3).

The time required for complete dephosphorylation of inositol hexa-P was 3 days in lettuce seeds (9) and 6 days in oat seeds (1). The insignificant change in concentration of inositol hexa-P during 48 hours suggests that phytase activity in crested wheatgrass seeds is low.

The concentration of P_i was 16 μmoles per g dry weight initially and decreased to 12 μmoles during the first 12 hours. Measurements of phosphate in the germinating dish indicated that 2 μmoles phosphate had diffused from seeds. The remaining 2 μmoles were incorporated into phosphate esters and into the phosphate fraction that was insoluble in cold aqueous trichloroacetic acid.

Acknowledgment

We are grateful to Edward Scott and Paul Madsen for assistance in making phosphate determinations.

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