Glucose Metabolism and Retention of Glucose Metabolites in the Wheat Embryo during Early Germination

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ABSTRACT

There is a lag period in the growth of excised wheat (Triticum vulgare Host.) embryos extending from 0.5 hour to 4.5 hours of their imbibition in water. During this time there is a sharp increase in the embryos' capability to retain several amino and organic acids, to synthesize cell wall components and starch, and to take up glucose. Their capability to metabolize glucose also increases by 30%. Elevation of the ATP content of 1-hour embryos by incubation in 3 mM adenosine is not sufficient to bring about these changes. These changes may be a part of a metabolic adjustment in the embryos which increases their growth potential.

Imbibition in water is sufficient to initiate the germination of wheat seeds as well as of isolated wheat embryos. Isolated embryos double their fresh weight after 30 min of imbibition, remain at approximately the same weight for the next 4 hr, and then resume growth (11). Pronounced increases in the rates of total protein synthesis and RNA synthesis are observed during the first hours of imbibition (14). An obvious question is whether any specific biochemical changes are differentially activated in the embryo during this period which may be considered prerequisite for later developments, or, whether the lag in growth during the first hours is caused by lack of some essential components which become sufficiently available later. In an attempt to screen for such possible changes, [14C]glucose was supplied to isolated wheat embryos in tracer amounts after different periods of imbibition, and its distribution was followed. A significant change in distribution of the label should indicate a shift in the activity of some glucose-utilizing pathway(s). The possible impact on growth by such changes and some possible ways by which they may be regulated in vivo are considered. During the experimental work it also became apparent that the embryos' ability to retain various metabolites in their cells dramatically increases during the period 1 to 3 hr after imbibition. Possible causes for these changes are also explored.

MATERIALS AND METHODS

Plant Material. Wheat (Triticum vulgare Host.) embryos of the cvs. Fortuna (1973 harvest) and Norana (1974 harvest) were isolated from seeds by grinding in a blender, sieving and floating on mixtures of cyclohexane and carbon tetrachloride 1:1.75 to 1:2.5 (v/v) according to Marcus et al. (12). Batches of 125 mg of embryos were imbibed in 1.6 ml of water or test solution on three discs of Whatman No. 1 filter paper in 5-cm Petri dishes. For incubation with [14C]glucose, embryos were transferred to 25-ml Erlenmeyer flasks containing 1.5 ml of incubation medium and shaken at a low speed. A stream of air was passed through the flasks and bubbled through tubes containing 10 ml of 33% methanolic solution of hyamine hydroxide.

Materials. [14C]Glucose, uniformly labeled, 199.6 µCi/µmol, was purchased from New England Nuclear; hexokinase from Boehringer; glucose-6-P dehydrogenase, P-glucose isomerase, ATP, ADP, and NADP from Sigma; precoated 0.1-mm MN-cellulose 300 plates for TLC from Brinkmann; AG 1-X10, 100 to 200 mesh, CI- form and AG 50W-X2 100 to 200 mesh H+ form ion exchange resins from Bio-Rad. Other chemicals were from different commercial sources.

Extraction and Identification. After incubation with the label the embryo samples were washed twice with 3 ml of distilled H2O, and the wash was combined with the incubation medium. The embryos were then rewashed under low suction, blotted, and ground for 2 min in 2.5 ml of boiling 80% (v/v) ethanol, using a motor-driven Teflon pestle and 15-ml Corex tubes. The precipitate was ground again in 2.5 ml of boiling 50% ethanol and the extracts were combined. The residue was collected in 5 ml of 0.1 N NaOH and ground for 2 min. The dissolved fraction contained proteins, RNA, lipids, and other unidentified compounds. The precipitate, containing starch and cell wall material, was collected in 4 ml of water, boiled for 30 min, cooled to room temperature, brought to a concentration of 14% (v/v) perchloric acid in a total volume of 5 ml, and the starch was extracted according to Hassid and Neufeld (6). The remaining precipitate, including cell wall material, was collected in 5 ml of water. Precipitation after each extraction step was carried out in a Sorvall centrifuge at 15,000 rpm for 10 min.

The ethanol-soluble fraction and the combined wash and incubation medium were each fractionated on AG 50-H+ form and AG 1-formate form ion exchange columns (1 × 9 cm) into amino acid, organic acid, and neutral fractions. Amino acids were further separated into acid and neutral fractions using a AG 1-acetate form column (1 × 9 cm) following Hirs et al. (8). Trinitiated alanine and glutamic acid were used as internal markers in some of the separations. The components of each fraction were then identified by TLC according to Haworth and Heathcote (7). The organic acid fraction was applied to an AG 1-formate form column (1 × 14 cm), and eluted with 400 ml of a linear gradient of 0 to 6 N formic acid at a rate of 0.9 ml/min. Fractions of 3.6 ml were collected and the radioactivity in them was determined by scintillation counting. Thus, four distinct peaks were resolved. Fractions in each radioactive peak were pooled together. Each of these was further analyzed by TLC on cellulose plates using phenol-water-formic acid (75:24:1) and the organic phase of the mixture 1-butanol-formic acid-water (100:30:100) as solvents. The identity of malic, citric, succinic, and fumaric acids was confirmed using a cellulose column according to Varner (16). Sugar phosphates

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in fraction III were identified by TLC according to Bandurski and Axelrod (1) and by ion exchange chromatography of their borate complexes according to Khym and Cohn (10).

Radioactivity was determined by scintillation counting. Non-radioactive compounds used for co-chromatography were sprayed with ninhydrin for amino acids or bromothymol blue for organic acids, following Stahl (15).

The neutral fraction was chromatographed with 1-butanol-ethanol-water (50:32:18) and with 1-butanol-acetic acid-water (4:1:5). All of the radioactivity in this fraction co-chromatographed with \( ^{14} \text{C} \) glucose.

ATP and ADP were determined according to Cheung and Marcus (3). Glucose and fructose were determined spectrophotometrically according to Stein (13) with hexokinase, P-glucose isomerase, and glucose-6-P dehydrogenase. Glucose-6-P and fructose-6-P were determined according to Horohost (9).

Hexokinase Determination. Batches of 125 or 250 mg of embryos were ground in 10 to 20 times their volume in a glass homogenizer on ice in the following grinding medium: triethanolamine-HCl buffer (pH 7.6), 50 mm; Na-EDTA 2 mm; 2-mercaptoethanol, 0.1%; glucose, 0.1 m; sucrose, 0.4 m. Homogenates were centrifuged in the cold at 2,500 rpm for 10 min and the precipitate was discarded. They were then fractionated into a mitochondrial preparation and a supernatant by centrifugation at 10,000 rpm for 10 min. Hexokinase was assayed spectrophotometrically at 340 nm at 25 C. Each reaction mixture contained, in a final volume of 1 ml: triethanolamine-HCl (pH 7.6), 80 mm; glucose, 1 mm; MgCl\(_2\), 5 mm; ATP, 2 mm; NADP, 0.5 mm; glucose-6-P dehydrogenase, 0.5 unit. One unit of hexokinase will catalyze the phosphorylation of 1 mmol of glucose/min under the above conditions. All of the experiments described in this paper were repeated at least three times, with similar results. Tables I through V present the results of these experiments.

RESULTS

Embryos were imbibed in water for periods of 0.5 hr, 2.5 hr, and 6.5 hr, then given a 20-mm pulse of 5 mm uniformly labeled \( ^{14} \text{C} \) glucose (199.6 \( \mu \)Ci/ml), or a pulse followed by a chase period of 20 min in distilled H\(_2\)O. The uptake of glucose and the distribution of its radioactive metabolites as well as their retention in the embryos after different periods of imbibition are compared in Table I. Salient features of the changes in glucose metabolism during the early hours of germination are as follows. (a) Glucose uptake increases 2-fold by 3 hr and 4-fold by 7 hr. (b) The utilization of labeled glucose in the tissue increases from 54% at 1 hr to 85% at 3 hr, and remains at that level by 7 hr. (c) The amount of labeled glucose incorporated into starch and cell wall increases considerably between 1 hr and 3 hr, and then decreases to a level higher than that of 1-hr embryos. In parallel with the drop in incorporation into polysaccharides there is an increase in incorporation into amino acids, particularly alanine (Table II). (d) One-hr embryos leak 50% of their radioactive metabolites into the surrounding solution. This drops to 10% at 3 hr. The relative amount of labeled glucose found in the chase solution also drops with time of imbibition.

The distribution of different metabolites between the embryos and the outside solution—using embryos after different periods of imbibition—is compared in Table II. Evidently some metabolites, such as the acidic amino acids, are retained in the tissue better than others. The ability of the embryos to retain different metabolites—expressed by the ratio in/out—increases with time of imbibition; however, this increase is different for each group, and acidic fraction I still leaks considerably at 7 hr. It is also observed (see Table IV, lines 9–12) that the retention of organic acids, but not of amino acids, is energy-dependent. I concluded that different mechanisms must be responsible for the ability of the embryos to retain different metabolites.

One possible explanation for the high leakage of 1-hr embryos is that their cell membrane is physically damaged during their preparation by floating on organic solvents, and that this damage is repaired with time of imbibition. To test this possibility, the experiment shown in Tables I and II was repeated using embryos prepared by a different method, namely by floating on 1.8 m sucrose according to Spiegel et al. (14). In the sucrose-prepared embryos, 44%, 12%, and 8% of the glucose metabolites leaked out of 1-hr, 3-hr, 7-hr embryos, respectively. This resembles the situation in embryos prepared by organic solvents. Moreover, the distribution of the different groups of metabolites between the embryos and the outside solution was also similar in both types of embryos (data not shown).

An apparent "leaking" could be observed if the glucose metabolites in the outside solution were produced by surface-associated bacteria rather than by the embryos. Fifty-mg batches of embryos were incubated on 1% agar in 5-cm Petri dishes at 25 C for 48 hr and checked for the appearance of microbial contamination. No such contamination was observed. The experiment described in Tables I and II was repeated using sterile dishes and media, with results similar to those obtained under nonsterile conditions. Finally, the extent of possible microbial activity could be expected to be greater on embryos imbibed for longer periods. In fact, the opposite was observed.

The above evidence favors the possibility that the increased capability to retain glucose and its metabolites in the embryos results from the development of specific mechanisms for this purpose such as compartmentation in the cell or active transport across the cell membrane. This increased capability to retain metabolites obviously contributes to the embryos' growth potential.

The basis for the analytical approach using labeled glucose is the expectation that the exogenous tracer mixes with the internal...
glucose pool and thereby serves to monitor processes occurring within the embryo. As a partial test of this point, the incorporation of [14C]glucose was tested with 1-hr and 3-hr embryos incubated for 0.5 hr in a 0.16 mM glucose solution. At this high concentration, the uptake by 1-hr embryos was 6.4 μmol and by 3-hr embryos 9.2 μmol, which exceeded their internal glucose concentration 4- and 8-fold, respectively (Table III). This experiment can also serve to measure the embryos' potential to metabolize glucose, because under these conditions glucose availability does not limit the hexokinase reaction. One-hr and 3-hr embryos metabolized 2.4 μmol and 3.5 μmol of glucose, respectively, during 0.5 hr. The distribution of the labeled metabolites in both 1-hr and 3-hr embryos was nearly identical to that in embryos incubated with tracer amount of glucose (data not shown). This suggests that mixing with the internal pool does not change the fate of externally administered glucose.

Having established the difference between 1-hr and 3-hr embryos in their capacity to metabolize glucose, I sought to test possible causes which might regulate it, assuming that the site of regulation may be the hexokinase reaction, which is the first step in the metabolism of glucose. Table III presents data on the determination of a number of pertinent components. These include the level of hexokinase activity in vitro in the supernatant and in the mitochondria; pellet, and the levels of glucose, fructose, and their 6-phosphates. No differences were found in these levels between 1-hr and 3-hr embryos, except for a decline of 33% in glucose and fructose content by 3 hr. Another possible regulatory component which could affect glucose metabolism is the concentration of ATP, which increases by 50% between 40 min and 3 hr of imbibition (C. P. Cheung and A. Marcus, unpublished). Two approaches were used to modify the level of ATP in the embryos, and glucose metabolism was examined under these modified conditions to test the possibility of a relationship between the level of ATP, the rate of glucose metabolism, and the capacity to retains glucose metabolites. In one approach a 1-hr situation with respect to ATP level was simulated in 3-hr embryos by adding 1 mM DNP during the last 30-min incubation assay (Table IV), or by incubation in a 5 mM KF solution throughout the experiment (Table V). In the latter approach, 3 mM adenosine was included throughout the imbibition and incubation periods of 1-hr embryos, resulting in a 30% increase in the ATP level in 1-hr embryos, thus approaching the ATP level of 3-hr embryos. Such an effect of adenosine on ATP level was reported by Grummit and Grummit (5) in Ehrlich ascites cells. It is observed (Table V, line 4) that a reduction of ATP level in 3-hr embryos by KF is correlated with a decrease in glucose metabolism. This is not obtained if DNP is used (Table IV). Moreover, a high level of ATP in the tissue per se is not sufficient to increase the rate of glucose utilization by 1-hr embryos. Polysaccharide biosynthesis and the retention of some metabolites are inhibited by both KF and DNP, but the availability of ATP in 1-hr embryos is not sufficient to induce polysaccharide biosynthesis, and its effect on the retention of organic acids is slight (Table IV). Of the parameters examined in Table V (using 3-hr embryos), utilization of glucose was inhibited by the lowest concentration of KF tested (1 mM). At 2 mM KF, inhibition of glucose incorporation into polysaccharides is also observed. Glucose uptake is only inhibited by 5 mM KF. Increased leaking of organic acids is observed between 2 mM and 10 mM KF and leakage of amino acids begins when KF concentration is 10 mM. If this order of inhibition reflects the sensitivity of these reactions to the availability of ATP, it may serve as a clue to the understanding of the order of their activation between 1 hr and 3 hr.

**DISCUSSION**

Glucose level in the tissue—1 μmol/125 mg of embryos—is low in comparison with the embryos' capacity to metabolize it (2.4-3.5 μmol/125 mg 0.5 hr). It remains fairly constant during the first hours of germination (Table III). Glucose in the endogenous pool must therefore have a short half-life, in the order of 10 min. Another conclusion, based on the increase in the rate of glucose metabolism with time of germination as well as the high turnover of the pool, is that the pool must be constantly replenished by mobilization of some reserve, and that the rate of reserve mobilization must be regulated in a process linked to the utilization of glucose.

Part of the increase in glucose utilization is channeled into anabolic pathways (Table I), in particular the synthesis of cell wall components and starch. This may constitute a part of a metabolic adjustment taking place during the early hours of imbibition, so as to enable cell expansion to commence at 4.5 hr.

The availability of glucose to cereal embryos under natural conditions increases after a lag period necessary for the induction of hydrolyase production and action in the endosperm (4). The capability of dormant *Avena fatua* seeds to utilize sugars is reduced relative to nondormant seeds (2). It thus appears that an increase in the embryos' capacity to utilize glucose is part of their natural course of germination.

Another phenomenon which appears to contribute to the embryos' growth potential is their increased ability to retain several amino and organic acids. This phenomenon shows some features characteristic of active transport systems, namely, substrate specificity and demand for energy supply. However, so far no such systems have been characterized in plants, which makes it pref...
erable to approach this problem in more general terms. It is believed that the determination of the distribution of the metabolites of a natural substrate, followed in the present study, reflects the physiological situation better than the often used method of loading tissues with nonmetabolizable analogs.

The supply of ATP, either as a reactant or as a source of energy, is important for all of the phenomena described in the present article. However, the use of adenosine to increase its level in physiologically immature tissue suggests that more factors are necessary for overcoming the lag period during the growth of the wheat embryo.

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