Plant Physiol. (1992) 98, 1098–1104
0032-0889/92/98/1098/07/$01.00/0

Received for publication October 23, 1991
Accepted October 25, 1991

Endosperm Acidification and Related Metabolic Changes in the Developing Barley Grain

Peter K. Macnicol* and John V. Jacobsen

Commonwealth Scientific and Industrial Research Organization, Division of Plant Industry, Canberra, A.C.T., Australia

ABSTRACT

The starchy endosperm (SE) of the developing grain (caryopsis) of barley (*Hordeum vulgare* L.) cv Himalaya, as well as that of other barley cultivars examined, acidifies during maturation. The major decrease in pH begins with the attainment of maximum grain dry weight, onset of dehydration, and completion of chlorophyll loss. Acidification is correlated with the accumulation of malate and lesser amounts of citrate and lactate, produced and probably secreted by the pericarp/testa/aleurone (PTA). It is accompanied by large concurrent rises in phospho*enol*pyruvate carboxylase and alcohol dehydrogenase (ADH) activity in the PTA. The activity of seven other enzymes of oxaloacetate and pyruvate metabolism was found to fall or rise only slightly during acidification. Sequential changes in relative amount of ADH isozymes were found in both PTA and SE. The PTA maintained a high respiration rate and adenylate energy charge (AEC) throughout acidification, whereas the SE showed a low respiration rate and rising AEC. The data are consistent with the occurrence of hypoxia in the SE. It is suggested that the above enzyme changes are required for the development of a malate/ethanol fermentation (*i.e.* a mixed metabolism) in the aleurone layer during maturation.

The mature endosperm (comprising the SE[1] and surrounding aleurone layer) of barley and other cereals has been studied extensively with regard to its structure, composition, and function during germination (reviewed in ref. 14). However, much less is known about endosperm development, despite an accumulation of data on morphology, starch synthesis, storage protein deposition, and enzyme activities (5, 12, 31). This is especially true of the maturation process, which involves transition from grain-filling to preparation for quiescence, dormancy, survival under adverse conditions, and finally germination. Despite the charting of enzyme activities related to starch degradation, glycolysis, and the pentose phosphate pathway (reviewed in ref. 13), no integrated picture of endosperm metabolism, or of the relative roles of the aleurone and SE, is available.

It has been observed that ADH accumulates in the outer layers of the barley grain during development and that the synthesis of ADH in mature aleurone tissue is increased under low O_2 concentration (19). This suggested the possibility of hypoxic conditions within the developing grain. Because an early response of plant cells to hypoxia is acidification of the cytoplasm caused by lactic acid production (11, 30), we asked whether the endosperm of the developing barley grain also acidifies.

In this report, we show that a marked acidification does in fact occur in the SE, due chiefly to production and secretion of protons and malate by the aleurone tissue. A number of biochemical changes are found that shed light on the mechanism of this process, the possible role of hypoxia, and the development of fermentative pathways.

MATERIALS AND METHODS

Plant Material

Grain of barley (*Hordeum vulgare* cv Himalaya) was incubated at room temperature for 2 d on filter paper in 9 cm Petri dishes containing 5 mL water. The germinated grain was planted in 12 cm diameter pots containing a 50:50 mixture of perlite and vermiculite. For each crop, about 45 pots were planted, each pot containing two plants. Each plant was limited to three tillers.

Crops were raised in growth cabinets in the Canberra phytotron under a 16 h (18°C) day/8 h (13°C) night regimen. Irradiance during the day was 400 μmol m^{-2} s^{-1} at pot height (600 μmol m^{-2} s^{-1} at ear height) from a composite light source including 8 × 400 W metal arc lamps (Siemens Powerstat multimetal arc lamps), 2 × 500 W quartz iodide lamps, and 4 × 60 W standard incandescent lamps. The plants were watered each morning with nutrient solution (Hoagland No. 2 solution) and each afternoon with demineralized water. At anthesis, taken to be when the first anthers appeared (about 39 d after planting), each ear was tagged with the anthesis date. On each harvest, usually 5 d apart beginning at 10 or 15 DPA, about 20 ears were collected, about 20 central grains were removed from each ear, and the total grain sample was mixed well. The bulked grain was subsampled for each assay. The grains were either used intact, or the embryo and distal end were excised, giving "half-grains" so as to facilitate the stripping of the combined outer layer, the PTA, from the SE. For this operation, small spatulas were used. After 40 to 45 DPA (depending on the batch of plants), when stripping was no longer possible owing to desiccation of the grain, samples of SE were obtained by shaving off the PTA (with a small amount of adhering SE) using a sharp scalpel.

[1] Abbreviations: SE, starchy endosperm; ADH, alcohol dehydrogenase; AEC, adenylate energy charge; DPA, days post anthesis; PEPC, phospho*enol*pyruvate carboxylase; PTA, pericarp/testa/aleurone.

Fresh Weight and Dry Weight

Twenty whole grains were weighed immediately after harvest to determine fresh weight and these grains were then dried at 80°C for 2 d for determination of dry weight.

Chl Determination

Chl was assayed in two replicate samples of 10 whole grains using the method of Arnon (2).

pH Determination

Twenty PTA or SE were homogenized in a total of 3 mL distilled water using a mortar and pestle with acid-washed sand, and the pH of the unclarified homogenate was immediately measured.

Organic Acid Analysis

Three replicate samples of 20 SE were ground to a powder under liquid N_2 in a mortar. This was extracted with a total of 7 mL 80% methanol by further grinding with acid-washed sand. The homogenate was centrifuged at 20,000*g*, the supernatant was recovered, and the pellet reextracted with 3 mL 80% methanol. Half of the combined extract was subjected to cleanup on a 20 × 5 mm column of DEAE-Sephadex A-25 by the method of Chalmers and Watts (8) modified by the use of triethylammonium acetate buffer instead of pyridinium acetate. The eluate was taken to dryness in a SpeedVac concentrator (Savant), dissolved in 2 mL water, and passed through a 0.45 μm filter. Organic acids were separated by HPLC on two Bio-Rad HPX-87H columns connected in series (15) and detected at 214 nm.

Ethanol Determination

The tissue was dropped into ice-cold 1 N perchloric acid immediately on dissection. Ethanol was determined in two replicate samples of PTA and SE by the method of Bernt and Gutmann (4).

Enzyme Assays

In each case, two replicate samples of 10 PTA and SE were used. ADH was extracted and assayed as described by Hanson *et al.* (19).

The other enzymes were extracted with 100 mM Hepes-KOH, pH 7.5, containing 0.5% (w/v) BSA, 1 mM $MnCl_2$, and 10 mM DTT, by grinding with sand in a mortar. The brei was diluted with extraction buffer and centrifuged at 30,000*g* for 10 min. The supernatant was saved and the pellet reextracted with buffer. An aliquot of the combined extract was desalted and buffer-exchanged on a small Sephadex G-25 column (PD-10, Pharmacia) into 10 mM Hepes-KOH, pH 7.5, containing 0.5% BSA, 1 mM $MnCl_2$, and 1 mM DTT. Enzyme assays were conducted spectrophotometrically at 25°C by following reduced pyridine nucleotide absorption at 340 nm in a reaction volume of 1 mL.

PEPC was assayed essentially as described by Hatch and Oliver (22). The reaction mixture contained 50 mM Tris-HCl, pH 8.3, 2.5 mM PEP, 5 mM $NaHCO_3$, 5 mM $MgCl_2$, 1 mM glucose-6-phosphate, 4 mM DTT, 0.2 mM NADH, 2 units of MDH, and enzyme extract.

Pyruvate, Pi dikinase, and NADP-malate dehydrogenase were assayed according to Ashton *et al.* (3), NADP-malic enzyme by the method of Hatch and Mau (21), and NAD-malic enzyme according to Hatch *et al.* (23). The measured activity of NAD-malic enzyme was unaffected by adding 5 μm NADPH and was therefore not due to NADP-malic enzyme (21).

NAD-malate dehydrogenase was assayed in a reaction mixture containing 50 mM Hepes-KOH, pH 7.5, 0.5 mM EDTA, 0.2 mM NADH, and 1 mM oxaloacetate; pyruvate carboxylase in 100 mM Mes-NaOH, pH 6.5, 5 mM $MgCl_2$, 1 mM thiamine pyrophosphate, and 0.2 mM NADH; and lactate dehydrogenase in 50 mM Hepes-KOH, pH 7.2, 5 mM pyruvate, and 0.2 mM NADH. The participation of ADH in the measured lactate dehydrogenase rates was excluded by the fact that they did not change on the addition of 10 mM 4-bromopyrazole (28).

Separation of ADH Isozymes

ADH isozymes were separated by horizontal starch gel electrophoresis and detected as described by Hanson and Brown (17).

Respiration Rate

Oxygen uptake in the dark was measured at 25°C using the Warburg technique. Two replicate samples of 20 PTA or SE were placed in the flasks on polythene gauze above water drops to maintain humidity. The rate of O_2 uptake was constant for several hours after tissue preparation, and the sum of the rates for PTA and SE was close to that of the half-seed.

Adenine Nucleotide Analysis

Two replicate samples of 10 PTA and SE were dissected into liquid N_2, where they were stored prior to extraction. Each sample was powdered under liquid N_2 and then homogenized with 5 mL 5% (w/v) TCA, while thawing, using a Teflon/glass homogenizer. The PTA homogenate was treated with 150 mg Polyclar AT for 30 min at 0°C with frequent swirling to remove interfering polyphenolic material. Subsequent steps were done rapidly at 0 to 5°C. After centrifugation at 30,000*g* and reextraction of the pellet with 5 mL 5% TCA, the combined supernatant was extracted twice with 2 volumes of diethyl ether to remove TCA before being added to an equal volume of 100 mM Hepes-KOH, pH 7.5, containing 25 mM Mg acetate. Residual ether was removed by bubbling with N_2 and the extract made up to 20 mL with water. Adenine nucleotides were then assayed by a luciferase method (9).

RESULTS

Fresh Weight, Dry Weight, Chl, and pH of Endosperm

Figure 1 shows these parameters of grain development for a typical batch of Himalaya barley. Fresh weight peaked and

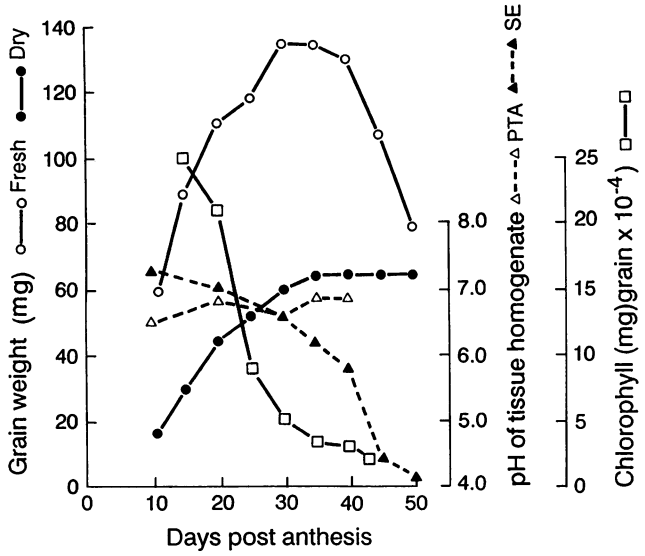

Figure 1. Fresh weight, dry weight, and Chl content of developing barley grain and pH of aqueous homogenates of isolated SE and PTA.

dry weight reached a plateau at 30 to 35 d, by which time chl had nearly disappeared. The pH of the PTA homogenate remained fairly constant at 6.5 to 7.0 up to 40 DPA; after this time, it was not possible to isolate the PTA cleanly (see "Materials and Methods") in this experiment. In contrast, the pH of SE homogenate decreased at first slowly to 30 DPA, then more rapidly, reaching about 4.2 at 50 DPA. The extent of the pH drop varied in different batches of plants, the final pH value being in the range of 4.0 to 5.2. Other cultivars of barley, namely Betzes, Clipper, and Proctor, showed similar trends in pH of the PTA and SE. The data in Figure 1 indicate that in Himalaya, 35 to 40 DPA is a critical time during which growth ceases, the grain desiccates, and acidification accelerates.

Organic Acids in SE

The acidification phenomenon caused us to look for accumulation of organic acids in the developing SE. Figure 2 shows that, beginning at 40 DPA, there was a large increase in malate accompanied by smaller increases in citrate and lactate. The levels of succinate, fumarate, and acetate did not increase during development. Two unidentified anions with retention times of 9.9 and 10.3 min decreased continuously in amount from 25 DPA onward. These and the above-named anions accounted for approximately 95% of the total peak area (214 nm absorbance) throughout development. Clearly, malic, citric, and lactic acids are the major organic acids involved in the rapid phase of acidification.

Can Organic Acids Account for the Acidification?

We next asked what contribution these three organic acids could make to the observed pH decrease in the SE. An aqueous homogenate was prepared (see "Materials and Methods") of SE at 30 DPA, *i.e.* before *in vivo* acidification; its pH was 6.6. When the amounts of malic, citric, and lactic acids present at 52 DPA (Fig. 2) were added to this homogenate, the pH decreased to 5.6. The pH at 52 DPA of SE from the same batch of plants was 4.8. The accumulation of the three acids, therefore, can account for over half of the observed pH decrease.

Role of the PTA in Acidification

An important question is whether the acid in the SE originated there or whether it was produced in adjacent tissues and secreted into the SE. Grains were harvested at 33 DPA (during the early stages of acidification) and half-grains, PTA, and SE were prepared and incubated on moist filter paper in Petri dishes. After 48 h, the half-grains had acidified to below pH 5, the pH of the isolated SE and its dish liquid had fallen very little, but the PTA had acidified the dish liquid to pH 3.4. HPLC analysis of this liquid showed a high concentration of malate, with some citrate and lactate. These results indicate that acidification can occur in the absence of the embryo and that the PTA is apparently the site of acid production, because PTA acidified the bathing liquid but the SE did not. Although in half-seeds the SE is acidified, it would appear that the acids are produced in the PTA and then released into the SE. We do not yet fully understand why acid production occurred at

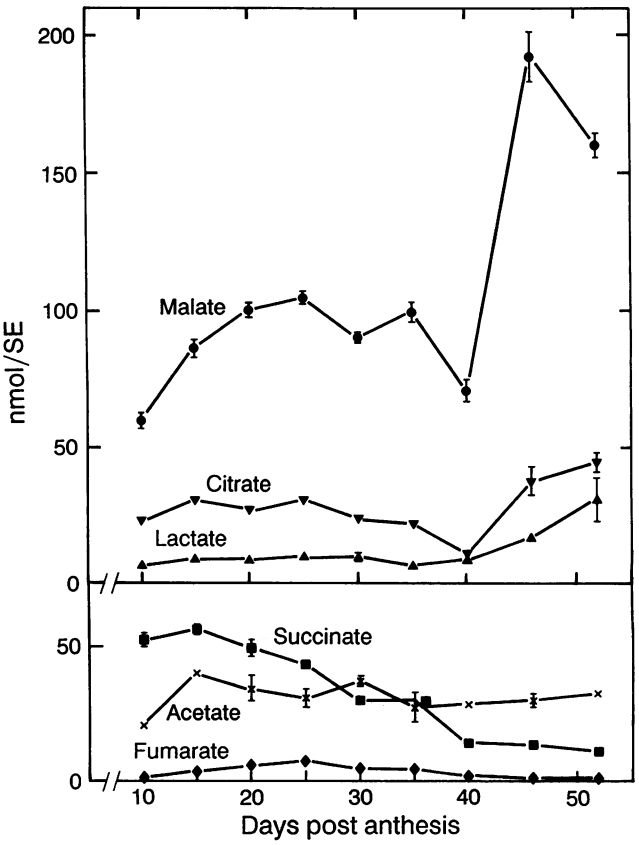

Figure 2. Organic acid levels in SE of developing barley grain. Error bars show SEs of three replicates; small SEs are contained within the symbols.

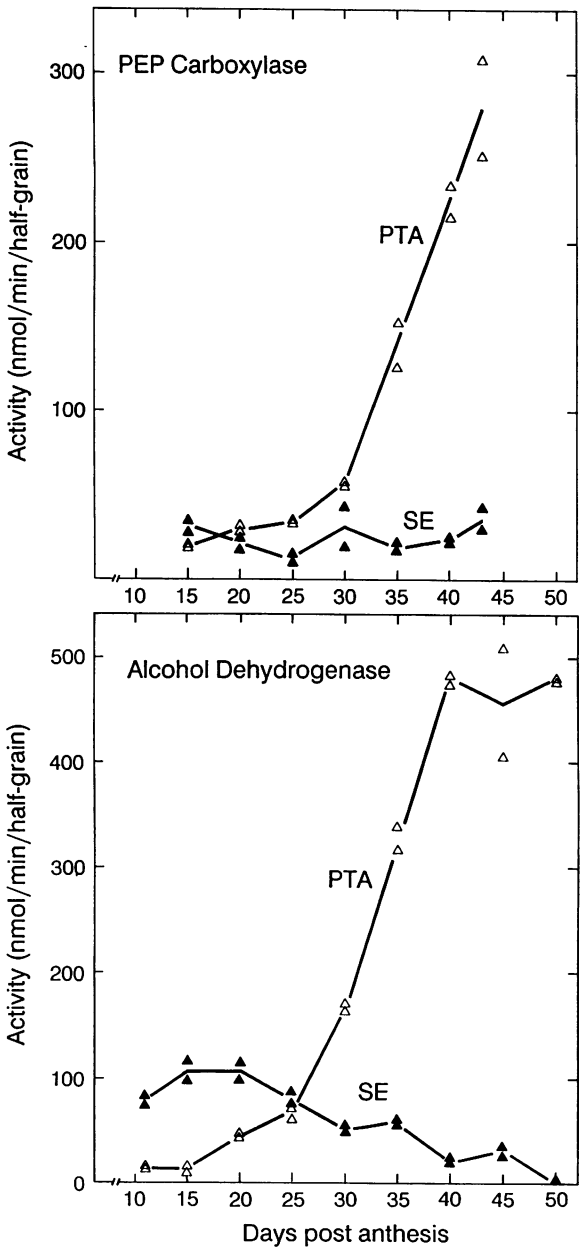

Figure 3. Extractable activity of PEP carboxylase and ADH in PTA and SE of developing barley grain. Results for two replicate tissue samples are given at each time point.

an accelerated rate in half-grains and PTA compared with grain left on the plant.

Enzyme Activities

The accumulation of malate in the endosperm led us to look for changes in enzyme activities related to oxaloacetate and pyruvate metabolism during the acidification phase. As shown above, this phase lasts from about 30 DPA to at least 50 DPA, but after 40 to 45 DPA, clean samples of PTA can no longer be obtained for enzyme assay.

Assay of desalted extracts showed large simultaneous rises in PEPC and ADH activity in the PTA, but no change or a slight fall in the SE (Fig. 3). This data for ADH extends that of Hanson *et al.* (19) to later development times. Rises in PEPC and ADH of the same extent and with the same developmental timing have been found in all of five separate experiments. Although the rises commence at around 15 DPA, the greatest rate of activity increase is during the acidification phase. During this phase, other enzyme activities in the PTA (data not presented) underwent a small rise (twofold: NAD-malic enzyme and pyruvate decarboxylase) or a fall (three–sixfold: pyruvate, Pi dikinase, NADP- and NAD-malate dehydrogenases, NADP-malic enzyme and lactate dehydrogenase) during acidification. The activity of these enzymes in the SE either fell or did not change, and was a minor proportion (5–35%) of total endosperm activity. NAD-malate dehydrogenase activity was always approximately 100-fold higher than that of PEPC.

ADH Isozymes

Barley contains three genes for ADH (17, 20). It is a dimeric enzyme and the polypeptide products of the three genes designated *ADH1, ADH2,* and *ADH3* (19) combine with each other to form all six possible dimers. The six dimeric isozymes can be separated from one another by electrophoresis as can be seen in Figure 4.

As shown in Figure 3, most of the ADH in the grain in the early stages of development (up to 20 DPA) was in the SE, and Figure 4 indicates that it was composed of ADH1, 2, and 3 polypeptides (the ADH2 and 3 homodimers probably did not appear because of their combination with the overwhelming amount of ADH1 polypeptide). As the ADH level decreased in SE from 20 DPA onward, the major loss appeared to involve ADH1, whereas ADH2 homodimer appeared transiently at 25 to 35 DPA. Subsequently, all isozymes appeared to decrease equally. In PTA at 15 DPA, ADH1 dominated the profile but, from then on, ADH2 and 3 polypeptides appeared. At 30 DPA and later, when the total ADH level had achieved its maximum rate of increase (Fig. 3), ADH2 and 3 (even the homodimers) became prominent in the isozyme profiles, although at 50 DPA there was less ADH3 polypeptide than at 45 DPA (Fig. 4). Similar isozyme patterns were obtained in two experiments.

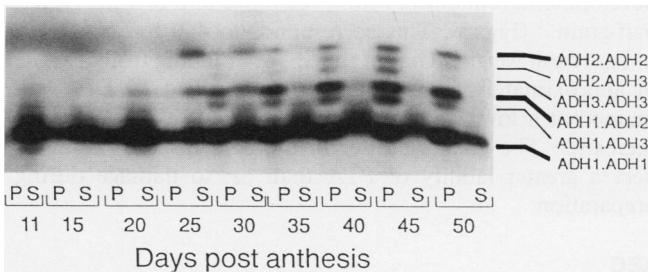

Figure 4. Starch gel electrophoresis of ADH isozymes in PTA (P) and SE (S) of developing barley grain. Each lane was loaded with extract equivalent to two grains. The diagram on the right identifies the isozymes.

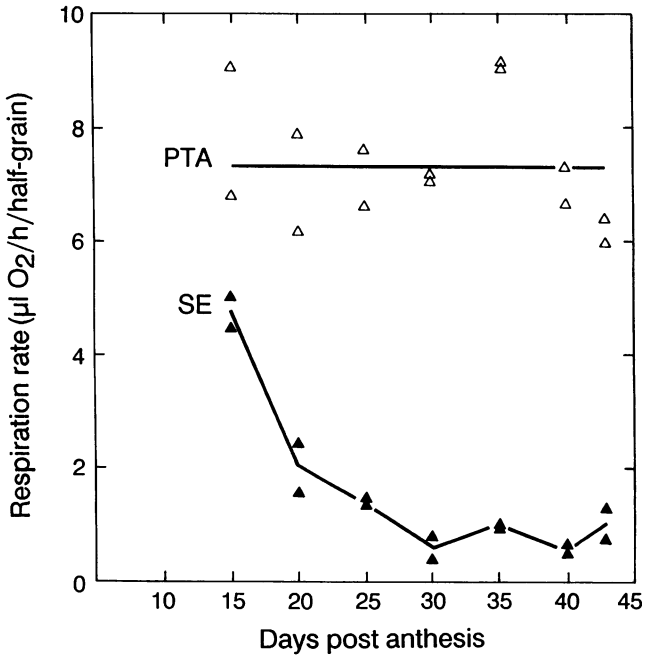

Figure 5. Dark respiration rate of PTA and SE during development of the barley grain. Results for two replicate tissue samples are given at each time point.

Ethanol Levels

The strong developmental rise in ADH activity in the PTA raised the possibility of an accumulation of ethanol in the grain. Throughout the period 15 to 45 DPA, the ethanol content of both PTA and SE remained relatively constant at approximately 500 nmol/half-grain (data not shown). Taking an average fresh weight of 20 mg/PTA and 50 mg/SE, and water content of 50%, this corresponds to about 50 mM ethanol in PTA and 20 mM in SE.

Respiration Rate

To see whether the apparent increase in fermentative metabolism of the endosperm during acidification was at the expense of aerobic metabolism, it was of interest to determine the capacity of the tissues for aerobic respiration. The respiration rate of the PTA did not change significantly during development, remaining within the range of 6 to 9 μL O_2 h^{-1} half-grain^{-1} (Fig. 5). This corresponds to 440 to 560 μL O_2 h^{-1} g^{-1} fresh weight. That of the SE, after falling from a value of approximately 5 μL O_2 h^{-1} half-grain^{-1} at 15 DPA, remained at a low value of 0.7 to 1.5 μL O_2 h^{-1} half-grain^{-1} thereafter. The greater scatter in the data for PTA may reflect a greater lability of PTA than SE to damage during preparation.

AEC

This parameter is defined as (2 ATP + ADP)/2(ATP + ADP + AMP) (see for example ref. 29). In the PTA, it remained close to 0.8 throughout development (Fig. 6). In the SE, however, it sank from an initial value of approximately 0.8 at 15 DPA to a low value of approximately 0.5 at 25 to 30 DPA, then increased steadily during the acidification phase to reach 0.8 again at 43 DPA. Over the whole period, total adenylates decreased from 3.8 to 2.3 nmol/half-grain in the PTA and from 13.8 to 1.8 nmol in the SE (Fig. 6).

DISCUSSION

The maturation phase of barley endosperm may be considered to begin with the loss of pericarp Chl, attainment of maximum dry weight, and onset of desiccation, which occur in our phytotron-grown material at 30 to 35 DPA. We have shown that 35 DPA is the beginning of a phase of rapid acidification of the SE of barley to a pH of approximately 4.5. Such a developmental acidification has not been reported previously, although the SE of germinating barley (7, 27) has been found to maintain a pH of 4 to 5, apparently via malic acid production by the PTA (27).

Our data suggest that the developmental acidification of the SE is due to the production of malic acid plus lesser amounts of citric and lactic acids by the PTA and their accumulation in the SE. This conclusion is based on the following points: first, the accumulation of malate, citrate, and lactate in the SE (Fig. 2) coincides with the rapid phase of acidification (Fig. 1). Second, the accumulated amounts of these acids are sufficient, when added to aqueous SE homog-

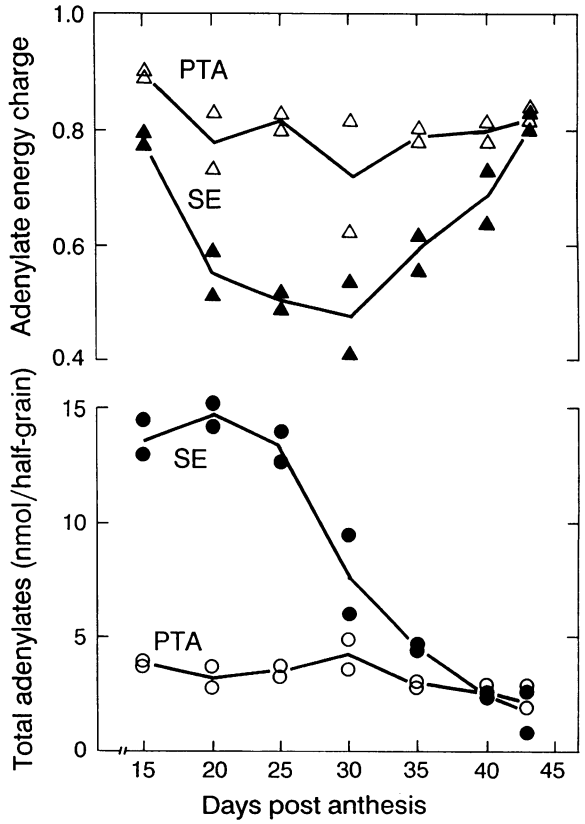

Figure 6. AEC and total adenylate pool of PTA and SE during barley grain development. Results for two replicate tissue samples are given at each time point.

enate, to bring about the greater part of the pH drop observed *in vivo*. Some of the pH decrease *in vivo* may also be caused by an age-dependent loss of buffering capacity in the SE due to loss of cell viability, as discussed below. Third, isolated PTA but not SE can acidify moist filter paper and secrete malic acid. Fourth, the activity of PEPC, an important step in malate synthesis, increases manyfold in the PTA but not SE from 30 DPA onwards (Fig. 3); this is considered in more detail below.

In what region of the PTA are the acids produced? Surgical isolation of the aleurone layer is not feasible, except at early developmental stages, so this question cannot be tested directly. However, two arguments suggest strongly that the aleurone is responsible. Anatomical studies of the developing barley grain (26) indicate that by 31 DPA the testa has been crushed and degeneration of the inner and outer pericarp cells is complete. Hence, the aleurone is likely to be the only metabolically competent tissue remaining in the PTA after this time. Isolated PTA of germinating mature barley, for which this is certainly true, has been shown by Mikola and Virtanen (27) to secrete malic acid into the incubation medium. In the developing grain, the presence of abundant plasmodesmata connecting the aleurone cells to those of the SE (31) would provide a path for transport into the SE.

The simplest explanation for our results is that the organic acids are synthesized in the aleurone cells and transported or secreted into the SE. It is equally possible that protons are pumped into the SE by the PTA, accompanied by passive or facilitated diffusion of malate and other anions to maintain electrical neutrality. We cannot yet distinguish between these two possibilities.

In plant cells accumulating large amounts of malate, the major source of the required oxaloacetate is usually the PEPC reaction (25). The large rise in activity of this enzyme in the PTA (Fig. 3), together with the excess activity of NAD-malate dehydrogenase, suggests that the increased PEPC activity is required for acidification. Yet, comparison of assayable PEPC activity with the rate of malate accumulation does not support the idea that the former limits the latter. Thus, the activity at 25 DPA, *i.e.* prior to rapid acidification, is 33 nmol min^{-1} half-$grain^{-1}$ (Fig. 3), which is over 1000-fold in excess of the rate of malate accumulation from 40 to 46 DPA (0.014 nmol min^{-1} half-$grain^{-1}$; Fig. 2). However, neither the rate of glycolysis (*i.e.* rate of supply of PEP) nor the actual rate of malate production is known. Furthermore, whereas the PEPC is assayed under optimal, activating conditions, it may be working in the aleurone cytosol under very suboptimal (*e.g.* acidic) conditions, in which malate inhibition of the enzyme is favored (1).

ADH activity in PTA also showed a large developmental rise, concurrent with that of PEPC (Fig. 3). Although the ethanol level did not change significantly with time in either PTA or SE, only steady-state values were measured that do not take account of possible loss by evaporation from the grain surface. Any increase in the rate of ethanol production resulting from the increased ADH activity, therefore, could be masked. ADH polypeptides 1, 2, and 3 appeared sequentially in the PTA during development, polypeptides 2 and 3 becoming prominent from 30 DPA onward (Fig. 4). In view of the work of Hanson and Jacobsen (18) with isolated mature PTA, in which synthesis of the ADH polypeptides was promoted sequentially, in the same order, by decreasing O_2 concentration from 20 to 0%, this suggests that the PTA of developing grain is under increasing hypoxia, particularly after 30 DPA. The decrease in ADH3 at 50 DPA may then indicate that aeration improves in mature grain. The ADH isozyme patterns in SE (Fig. 4) differ from those in the PTA and suggest that the SE experiences hypoxia earlier than the PTA.

Anatomical studies support the idea that hypoxia may occur within developing grains. A number of cuticles are formed in the pericarp and testa of the barley grain by 20 DPA (10, 16) and they are thought to present a barrier to gaseous exchange between the atmosphere and the inside of the grain (10). Once Chl has disappeared at 30 to 35 DPA, the pericarp can no longer provide O_2 by photosynthesis (13), and the O_2 level will be depleted.

The results discussed so far indicate at least a partial redirection of metabolism in the maturing PTA into malate fermentation. This, plus the circumstantial evidence for hypoxia within both PTA and SE, led us to investigate their capacity for aerobic respiration and their energy status. The PTA was found to maintain a high respiration rate throughout development (Fig. 5), comparable to that of barley root or young wheat leaf (6). Consistent with this, the PTA maintained an AEC close to 0.8 (Fig. 6), a value typical of normoxic tissue (11, 29). The SE showed a very different trend. Prior to the acidification phase, the respiration rate fell continuously to about one-fifth of its initial value, accompanied by a matching fall in AEC to 0.5 (Figs. 5 and 6). The AEC then recovered progressively during acidification to 0.8, whereas respiration rate remained at a low level. The fact that the total adenylate pool at 43 DPA was only approximately 8% of its size at 15 DPA (Fig. 6) indicates a large contraction in metabolic volume of the SE. Whether this is shared by all the cells, or whether some cells lose viability, is not known.

These changes in AEC in the SE are reminiscent of those found in plant tissues placed in nitrogen (11): the AEC sinks from an initial value of 0.8 to 0.85 and stabilizes at 0.5 to 0.6 or lower, depending on the degree of hypoxia, before recovering again when fermentation sets in. Like the ADH isozyme data, they point to hypoxia in the SE before the acidification phase, as early as 20 DPA. On the other hand, the continuing high AEC in the PTA does not support the idea of hypoxia in that tissue, contrary to the isozyme profile. Nevertheless, it may be risky to extrapolate from studies on experimental anoxia, with a time scale in hours, to the developmental situation in which the time scale is in days. A malate fermentation in the PTA could provide a net gain of ATP if the substrate entered as glucose-6-phosphate [*e.g.* from starch via phosphorylase and phosphoglucomutase or from translocated sucrose via sucrose synthase, ADPG(UDPG)-glucose phosphorylase and phosphoglucomutase]. This could raise the AEC of at least the outer SE cells. However, other explanations are possible for the rise in AEC of the SE, such as a drop in ATP-utilizing processes or decreased competition for O_2 by a diminishing proportion of viable SE cells.

The results of this study have significantly extended our knowledge of events in the maturation phase of barley grain development. We have shown that the SE is progressively

acidified by malic acid (or protons plus malate) produced in the PTA, almost certainly in the aleurone cells. Some or all of this malic acid evidently persists in the mature grain, in which a high level has been reported (24). Possible uses of this malate accumulation in germination are the generation of an acidic environment for the action of endosperm hydrolases and scutellar transport systems (27) or, alternatively, the provision of a pool of immediately metabolizable substrate. The spectacular increase in PEPC activity appears to be part of a metabolic switch to a malate fermentation. It is presumably coupled to an increased rate of glycolysis, but this remains to be demonstrated. The function of the equally large rise in ADH activity is less clear, as changes in ethanol content were not found. Some level of ethanolic fermentation obviously occurs throughout development. Evidence for and against hypoxia in the PTA was found, but some degree of hypoxia in the SE is likely. Resolution of this question requires the development of methods to analyze the internal atmosphere of grains.

ACKNOWLEDGMENTS

We thank Dr. Malcolm Allen (Charles Sturt University) for the organic acid analyses, Dr. Tony Brown for running the ADH isozyme gels, Dr. Hal Hatch and Dr. Philippe Raymond for helpful discussions, and Rosemary Metcalf for careful preparation of experimental material.

LITERATURE CITED

1. **Andreo CS, Gonzalez DH, Iglesias AA** (1987) Higher plant phosphoenolpyruvate carboxylase. Structure and regulation. FEBS Lett **213:** 1–8
2. **Arnon DI** (1949) Copper enzymes in isolated chloroplasts. Polyphenol oxidase in *Beta vulgaris*. Plant Physiol **24:** 1–5
3. **Ashton AR, Burnell JN, Furbank RT, Jenkins CLD, Hatch MD** (1990) Enzymes of primary metabolism. *In* PJ Lea, ed, Methods in Plant Biochemistry, Vol 3. Academic Press, New York, pp 39–72
4. **Bernt E, Gutmann I** (1974) Ethanol. Determination with alcohol dehydrogenase and NAD. *In* H Bergmeyer, ed, Methods of Enzymatic Analysis, Ed 2, Vol 3. Verlag Chemie, Weinheim, pp 1499–1502
5. **Bewley JD, Black M** (1978) Physiology and Biochemistry of Seeds in Relation to Germination. Vol 1. Development, Germination and Growth. Springer-Verlag, Berlin
6. **Bidwell RGS** (1974) Plant Physiology. Macmillan, New York, p 126
7. **Briggs DE** (1968) α-Amylase in germinating, decorticated barley. 1. Amylase, conditions of growth, and grain constituents. Phytochemistry **7:** 513–529
8. **Chalmers RA, Watts RWE** (1972) The quantitative extraction and gas-liquid chromatographic determination of organic acids in urine. Analyst **97:** 958–967
9. **Ching TM, Ching KK** (1972) Content of adenosine phosphates and adenylate energy charge in germinating ponderosa pine seedlings. Plant Physiol **50:** 536–540
10. **Cochrane MP, Duffus CM** (1979) Morphology and ultrastructure of immature cereal grains in relation to transport. Ann Bot **44:** 67–72
11. **Davies DD** (1980) Anaerobic metabolism and the production of organic acids. *In* DD Davies, ed, The Biochemistry of Plants, a Comprehensive Treatise, Vol 2. Academic Press, New York, pp 581–611
12. **Duffus CM** (1987) Physiological aspects of enzymes during grain development and germination. *In* JE Kruger, D Lireback, CE Stauffer, eds, Enzymes and Their Role in Cereal Technology. AACC Inc, St Paul, MN, pp 83–116
13. **Duffus CM, Cochrane MP** (1982) Carbohydrate metabolism during cereal grain development. *In* AA Khan, ed, The Physiology and Biochemistry of Seed Development, Dormancy and Germination. Elsevier Biomedical Press, Amsterdam, pp 43–66
14. **Fincher GB** (1989) Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. Annu Rev Plant Physiol **40:** 305–346
15. **Frayne RF** (1986) Direct analysis of the major organic components in grape must and wine using high performance liquid chromatography. Am J Enol Vitic **37:** 281–287
16. **Freeman PL, Palmer GL** (1984) The structure of the pericarp and testa of barley. J Inst Brew **90:** 88–94
17. **Hanson AD, AHD Brown** (1984) Three alcohol dehydrogenase genes in wild and cultivated barley: characterization of the products of variant alleles. Biochem Genet **22:** 495–515
18. **Hanson AD, Jacobsen JV** (1984) Control of lactate dehydrogenase, lactate glycolysis, and α-amylase by O_2 deficit in barley aleurone layers. Plant Physiol **75:** 566–572
19. **Hanson AD, Jacobsen JV, Zwar JA** (1984) Regulated expression of three alcohol dehydrogenase genes in barley aleurone layers. Plant Physiol **75:** 573–581
20. **Harberd NP, Edwards KJR** (1983) Further studies on the alcohol dehydrogenases of barley: evidence for a third alcohol dehydrogenase locus and data on the effect of an alcohol dehydrogenase-1 null mutation in homozygous and in heterozygous condition. Genet Res **41:** 109–116
21. **Hatch MD, Mau S-L** (1977) Association of NADP- and NAD-linked malic enzyme activities in *Zea mays:* relation to C_4 pathway photosynthesis. Arch Biochem Biophys **179:** 361–369
22. **Hatch MD, Oliver IR** (1978) Activation and inactivation of phosphoenolpyruvate carboxylase in leaf extracts of C_4 species. Aust J Plant Physiol **5:** 571–580
23. **Hatch MD, Tsuzuki M, Edwards GE** (1982) Determination of NAD malic enzyme in leaves of C_4 plants. Plant Physiol **69:** 433–449
24. **Klopper WJ, Angelino SAGF, Tuning B, Vermeire HA** (1986) Organic acids and glycerol in beer. J Inst Brew **92:** 225–228
25. **Latzko E, Kelly GJ** (1983) The many-faceted function of phosphoenolpyruvate carboxylase in C_3 plants. Physiol Veg **21:** 805–815
26. **MacGregor AW, Dushnicky L** (1989) α-Amylase in developing barley kernels—a reappraisal. J Inst Brew **95:** 29–33
27. **Mikola J, Virtanen M** (1980) Secretion of L-malic acid by barley aleurone layers (abstract No. 783). Plant Physiol **65:** S-142
28. **Pryor A, Huppatz JL** (1982) Purification of maize alcohol dehydrogenase and competitive inhibition by pyrazoles. Biochem Int **4:** 431–438
29. **Raymond P, Gidrol X, Salon C, Pradet A** (1987) Control involving adenine and pyridine nucleotides. *In* DD Davies, ed, The Biochemistry of Plants, a Comprehensive Treatise, Vol 11. Academic Press, New York, pp 129–176
30. **Roberts JKM, Callis J, Wemmer D, Walbot V, Jardetzky O** (1984) Mechanism of cytoplasmic pH regulation in hypoxic maize root tips and its role in survival under hypoxia. Proc Nat Acad Sci USA **81:** 3379–3383
31. **Simmonds DH, O'Brien TP** (1981) Morphological and biochemical development of the wheat endosperm. *In* Y Pomeranz, ed, Advances in Cereal Science and Technology, Vol IV. American Association of Cereal Chemistry, St Paul, MN, pp 5–70