Phytochemistry, Vol. 21, No. 9, pp. 2165–2171, 1982.
Printed in Great Britain.

0031-9422/82/092165-07$03.00/0

CYTOPLASMIC pH OF MAIZE SCUTELLUM CELLS*

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(*Revised received* 22 *January* 1982)

Key Word Index—*Zea mays*; Gramineae; maize scutellum; cytoplasmic pH.

Abstract—The cytoplasmic pH of scutellum cells from 3-day-old maize seedlings was estimated from the distribution of 2, 4-dinitrophenol (DNP) between scutellum slices and bathing solution. Although DNP is a protonophore, it had a negligible influence on cytoplasmic pH when slices (0.5 g) were placed in a DNP solution (0.1 mM, 10 ml) buffered at pH 6.1 (20 mM Mes). Under these conditions, DNP equilibrated between slices and bathing solution in 20 min, and a constant distribution was maintained for at least 40 min. The use of DNP solutions of lower pH gave lower estimates of cytoplasmic pH: cytoplasmic pH estimates of 8.0, 7.9, 7.7 and 7.4 were obtained from DNP solutions of pH 6.1, 5.8, 5.5 and 5.0, respectively. Another weak acid, 5,5-dimethyloxazolidine-2, 4-dione (DMO), was used to check reliability of the DNP results. With DMO-^{14}C, a cytoplasmic pH of 7.8 was calculated. Incubation of scutellum slices in HCl or buffer (pH range 2–8) for 1–2.5 hr before placing them in the DNP-pH-measuring solution (pH 6.1) had no effect on the estimated cytoplasmic pH, which remained near 8.0. When mannose (an energy poison in scutellum cells) was added to the HCl or buffer, the estimated cytoplasmic pH fell to about 7.5, but it was not influenced by the pH of the mannose solution. It is suggested that the higher pH estimate (8.0) reflects a strong influence of the mitochondrical matrix, whereas the lower pH estimate (7.5) is that of the cytsol, mannose having dissipated the pH gradient between cytosol and mitochondrion.

INTRODUCTION

In recent years there has been much interest in the measurement of cytoplasmic pH, stemming largely from the recognition (due to Mitchell, e.g. [1]) that proton electrochemical gradients across membranes constitute an energy source that can drive active uptake and ATP synthesis. Three methods have been used to estimate cytoplasmic pH: distribution of weak acids between bathing solution and cell, insertion of microelectrodes into the cell, and ^{31}P nuclear magnetic resonance (^{31}P NMR). The weak acid distribution method is the one most commonly used, and the weak acid of choice has been 5, 5-dimethyloxazolidine-2, 4-dione (DMO). In the alga, *Nitella* [2], DMO and glass microelectrodes gave similar estimates of cytoplasmic pH. DMO also has been used to measure the cytoplasmic pH of *Chara* [3], *Chlorella* [4], and the blue-green alga, *Anacystis* [5]. There are only a few reports of the use of ^{31}P NMR for pH measurement. DMO and ^{31}P NMR gave similar results with *Escherichia coli* [6, 7]. Using ^{31}P NMR, the cytoplasmic pH of maize root tips was estimated to be 7.1 [8], which is in reasonable agreement with values obtained in other higher plant cells using DMO or microelectrodes [9–11]. However, few estimates of cytoplasmic pH in higher plants have been made.

In this paper, distributions of 2, 4-dinitrophenol (DNP) and DMO between bathing solution and maize scutellum slices are used to estimate cytoplasmic pH. First, because DNP is a protonophore, the effect of DNP on cytoplasmic pH was investigated, and proper conditions for its use in pH estimation were determined. Then it is shown that in both normal and energy poisoned scutellum cells cytoplasmic pH was essentially independent of external pH in the range of 2 and 8.

RESULTS

Distribution of DNP between bathing solution and scutellum slices

Buffered DNP solutions were added to slices at zero time, and portions of the bathing solutions were removed at intervals over a 60-min period for DNP assay. The decrease in DNP content of the bathing solution was considered to be equal to the DNP accumulated by the cells.

DNP equilibrated between bathing solution and slices in 10 min when the bathing solution initially contained 0.1 mM DNP at pH 5.0 (Fig. 1A). At equilibrium, the slices contained about 60% of the DNP,

*Florida Agricultural Experiment Station Journal Series No. 3404.

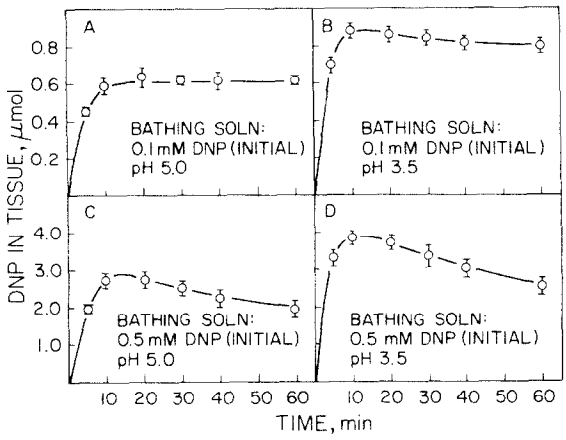

Fig. 1. Effect of pH and DNP concentration on the distribution of DNP between bathing solution and scutellum tissue. Data from four experiments.

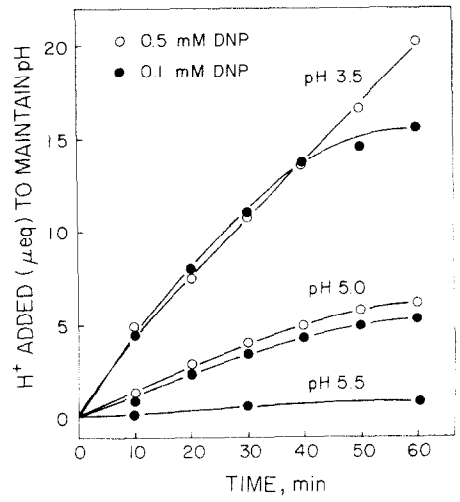

Fig. 2. Apparent proton influx during incubation of slices in DNP. Data are averages from two experiments.

and this distribution was maintained for at least 50 min. When the pH of the 0.1 mM DNP solution was lowered to 3.5, the DNP content of the slices reached a peak in 10 min and then slowly declined (Fig. 1B). When the DNP concentration was increased to 0.5 mM, the DNP content of the slices reached a peak in about 10 min at both pH 3.5 and 5.0, but the decline in DNP content was more rapid (Fig. 1C, D).

The efflux of DNP from the slices probably resulted from the acidification of the cell caused by DNP acting as a protonophore. The decline in DNP content from 10 to 60 min (Fig. 1) indicates a drop in cytoplasmic pH of 0.2 unit (0.1 mM DNP, pH 3.5 or 0.5 mM DNP, pH 5.0) to 0.6 unit (0.5 mM DNP, pH 3.5). The pH drop could be caused by protons entering the cytoplasm both from the bathing solution and from the vacuoles of these multivacuolate cells. Proton uptake from the bathing solution was measured as a function of pH and DNP concentration (Fig. 2). Initial rates of proton uptake at pH 3.5 were about three times greater than those at pH 5.0, but essentially the same rates of proton uptake were obtained during the first 40 min at initial DNP concentrations of 0.1 and 0.5 mM. However, at pH 5.5, 0.1 mM DNP caused little, if any, proton uptake (Fig. 2). From these results it appears that the drop in cytoplasmic pH, as shown by DNP efflux (Fig. 1B, D), could not have been caused only by proton uptake from the bathing solution, but also must have been caused by proton leakage from the vacuoles.

The constancy of the DNP distribution obtained with 0.1 mM DNP at pH 5.0 (Fig. 1A) indicates that these are at least minimum conditions necessary for measuring cytoplasmic pH with DNP. However, increasing the pH of the bathing solution decreases the amount of DNP in the cells, and thereby, should decrease the influence of DNP on cytoplasmic pH. The distribution of DNP between tissue and bathing solution in the pH range of 5.0–6.1 was measured using an initial DNP concentration of 0.1 mM (Fig. 3). As the pH was increased the time necessary for DNP to equilibrate between slices and bathing solution was increased from 10 min at pH 5.0 to 20 min at pH 6.1. Constant equilibrium distributions were maintained for at least 40 min in this pH range. If cellular DNP concentration did not affect cellular pH in the experiment of Fig. 3, the distribution ratio of the DNP anion (DNP_i^-/DNP_o^-) should be 12.5 times greater at pH 5.0 then at pH 6.1. However, only about a fivefold increase was obtained (Fig. 3), indicating that DNP was influencing cellular pH. Estimates were made of the extent of this influence of DNP on cellular pH in order to arrive at a suitable bathing-solution pH and DNP concentration for cytoplasmic pH determination.

Estimation of cytoplasmic pH using DNP

To calculate cytoplasmic pH from the distribution of a weak acid, it is necessary to know the cytoplas-

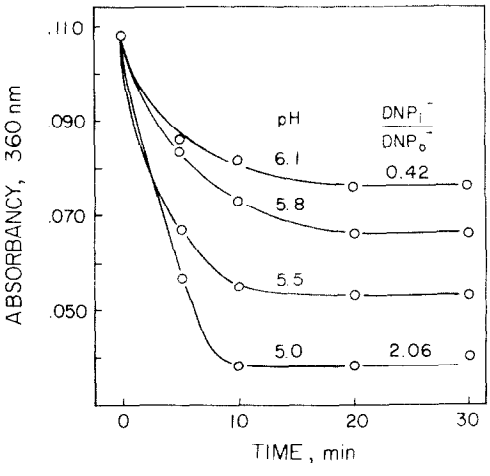

Fig. 3. Effect of pH on the time course of DNP influx. The initial DNP concentration was 0.1 mM. The DNP anion ratios are based on total amounts of DNP^- inside and outside, not on concentrations. Data from a single experiment but see Table 1.

mic volume, the vacuolar volume, and the vacular pH. Because the scutellum cells contain numerous small vacuoles, it was not possible to estimate the volumes of cytoplasm and vacuole by microscopic examination. Instead, the glucose space of the slices (see Experimental) was used as the cytoplasmic volume. Glucose space probably includes the nucleus [12] and glyoxysomes [13] but not mitochondria, plastids, spherosomes or vacuoles, and therefore, it is more nearly a measure of cytosolic volume than of cytoplasmic volume. However, underestimation of cytoplasmic volume by 20% results in an overestimate of cytoplasmic pH of less than 0.1 unit. Vacuolar volume was calculated by subtracting the glucose space from the volume corresponding to 55% of the fr. wt of the slices on the assumption that this wt represented water contained within the plasmalemma (dry wt of the slices was 30% of fr. wt). A rough estimate of vacuolar pH was obtained by killing slices (2 g) in boiling water (10 ml) and measuring the pH of the solution after it had cooled. In three experiments, a pH of 5.40 ± 0.02 was obtained, which can be regarded as an upper limit for vacuolar pH. If the true pH values of cytoplasm and vacuole were 7.5 and 5.4, respectively, an error of only 0.02 pH unit would be introduced into the estimate of cytoplasmic pH by ignoring the vacuole altogether and assuming that the DNP of the slices was entirely contained within the cytoplasm. Therefore, because estimates of cytoplasmac pH were usually 7.5 or above (see below) and because vacuolar pH was probably below 5.4, the vacuoles were ignored in calculating cytoplasmic pH from DNP distribution.

Cytoplasmic pH was estimated from the distribution of DNP (initially at 0.1 mM) between slices and bathing solutions buffered at pH 5.0, 5.5, 5.8 and 6.1 (e.g. Fig. 3), using the glucose space measurement at each pH to calculate the DNP-anion concentration of the cytoplasm. Estimates of cytoplasmic pH increased from 7.4 to 8.0 as the pH of the bathing solution increased from 5.0 to 6.1 (Table 1). At each bathing solution pH, DNP caused an increase in glucose space, but to a lesser extent at pH 6.1 (40%) than at pH 5.0 (60%). This effect of DNP on glucose space has been noted previously [14], and probably results from leakage of vacuolar solutes (e.g. sucrose [15]) into the cytoplasm followed by vacuolar shrinkage. However, the differences in glucose space did not cause the differences in the estimates of cytoplasmic pH. Recalculation of the DNP-anion concentrations of the cytoplasm in Table 1 using the glucose space found with DNP at pH 6.1 gave estimates of cytoplasmic pH of 7.5, 7.8 and 7.9 for DNP solutions of pH 5.0, 5.5 and 5.8, respectively. Therefore, the differences in cytoplasmic pH shown in Table 1 appear to be real; they are not due to vagaries in the estimates of cytoplasmic volume (i.e. glucose space), but undoubtedly resulted from differences in the amounts of DNP that entered the slices as the pH of the bathing solution was changed.

Since increasing the bathing solution pH from 5.8 to 6.1 changed the estimated cytoplasmic pH by only 0.1 units (Table 1), pH 6.1 and 0.1 mM DNP were the conditions used to estimate cytoplasmic pH in the experiments that follow.

Influence of external pH and mannose on cytoplasmic pH

External pH in the range of 4–8 has little effect on the ability of scutellum slices to synthesize sucrose from exogenous fructose [16], and it is therefore assumed that external pH has little effect on cytoplasmic pH. Either the plasmalemma is impermeable to protons and hydroxyl ions or systems are present that maintain cytoplasmic pH in the fact of leakage of these ions through the plasmalemma. The assumption that cytoplasmic pH is insensitive to external pH was investigated in the presence or absence of mannose. Mannose is an energy poison in scutellum cells, and should suppress systems that maintain cytoplasmic pH.

Slices were incubated in buffered solutions for 1 hr

Table 1. Effect of bathing solution pH on the distribution of DNP and on the estimated cytoplasmic pH of maize scutellum cells*

Bathing soln pH	Bathing soln DNP (μM)		Glucose space (μl/0.5 g)	Cytoplasmic DNP (mM)	Cytoplasmic pH
	Initial	Final			
5.0	0	0	43 ± 2	0	
5.0	100	38 ± 1	69 ± 2	9.00	7.4
5.5	0	0	41 ± 2	0	
5.5	100	50 ± 1	61 ± 3	8.20	7.7
5.8	0	0	40 ± 3	0	
5.8	100	61 ± 1	57 ± 2	6.84	7.9
6.1	0	0	40 ± 3	0	
6.1	100	70 ± 2	56 ± 1	5.36	8.0

*Slices were incubated in water for 30 min, rinsed once, and placed in buffered DNP for pH measurement (cf. Fig. 3) or in buffered glucose ± DNP for glucose space measurement (see Experimental). Cytoplasmic DNP concentration was calculated assuming all of the tissue DNP was uniformly distributed in the glucose space. Data are averages of 3–4 experiments followed by s.d.

at 30°, and then were washed and placed in 0.1 mM DNP at pH 6.1 for cytoplasmic pH measurement (cf. Table 1). External pH from 3 to 8 had no effect on cytoplasmic pH (Fig. 4). In one experiment, the length of the initial incubation in buffer was increased to 2.5 hr with the same result, an estimated cytoplasmic pH near 8.0 which was independent of external pH.

When mannose was added to the buffer solutions, cytoplasmic pH dropped to about 7.5 but remained independent of external pH (Fig. 4). As with the minus-mannose experiment, increasing the incubation time in buffered mannose to 2.5 hr had no effect. Mannose drops the ATP content of scutellum slices to less that 5% of that present initially [17]. It also strongly inhibits the plasmalemma proton pump [18], and the proton pump is thought to be one mechanism by which the cell maintains its pH in the face of a proton leak. The results with mannose indicate that the plasmalemma is highly impermeable to protons and hydroxyl ions in the pH range of 3–8 (see below).

The same constancy with time of DNP distribution was found with mannose-treated slices as was found with non-treated slices (cf. Fig. 3). Mannose treatment caused a doubling of the glucose space [14], and this was taken into account in calculating cytoplasmic pH. Mannose, DNP and triphenylmethylphosphonium ion ($TPMP^+$, a lipophilic cation) cause sucrose to leak from the vacuoles into the cytoplasm [14, 19], which presumably causes shrinkage of the vacuoles and an increase in glucose space. Because mannose as well as the liphophilic ions, DNP and $TPMP^+$, cause sucrose leakage, it appears that leakage results from low ATP levels and not from interaction of these compounds with the tonoplast.

Scutellum slices are resistant to treatment with HCl at pH 2. Enzymes at the cell surfaces such as invertase, maltase and acid phosphatase are inactivated, but HCl treatment does not influence respiration, sucrose synthesis, transport of sucrose and maltose across the plasmalemma or plasmalemma ATPase activity [15, 20–22]. Undoubtedly HCl at pH 2 has little effect on cytoplasmic pH, and this conclusion was verified by direct measurement (Table 2). Cytoplasmic pH was lowered by less than 0.1 unit following incubation of slices in HCl (pH 2) ±mannose for 60–90 min (cf. Table 2, Fig. 4).

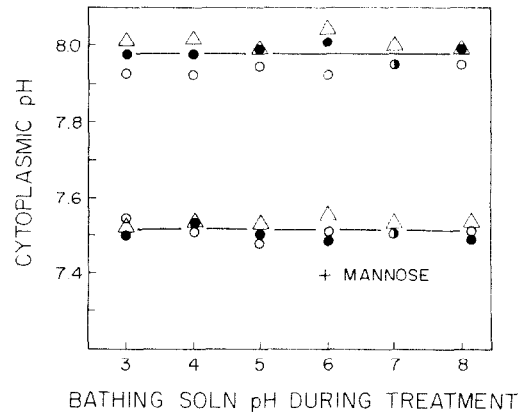

Fig. 4. Effect of bathing solution pH on cytolasmic pH. Data from three experiments.

During incubation in HCl, sucrose leaked into the bathing solution (Table 2). At an external pH of 8, sucrose efflux from maize scutellum slices occurs by a sucrose–proton co-transport system, one proton being transported with each sucrose molecule [16]. If the same co-transport system operates at pH 2, proton efflux equivalent to the measured sucrose efflux must have occurred in the experiments of Table 2. Sucrose–proton efflux is electrogenic [16] and would require a compensating influx of protons or efflux of anions. Since there was essentially no change in the estimate of cytoplasmic pH following HCl treatment, a proton influx of the same magnitude as the sucrose–proton is assumed, i.e. about 14 nmol/m^2 per sec. This assumption does not take into account the buffer value of the cytoplasm or the possibility that protons were secreted into the vacuoles (when mannose was present, proton secretion was unlikely). A passive proton influx of about 100 nmol/m^2 per sec was calculated for *Chara corallina* at an external pH of 4 [23], and the *Chara* cells did not survive prolonged exposure at this pH. The results with

Table 2. Effect of incubation in HCl (pH 2) on cytoplasmic pH of maize scutellum cells*

Time in HCl (min)	Mannose (50 mM)	Bathing soln pH		Cytoplasmic pH	Sucrose efflux† (nmol/m^2 per sec)
		Initial	Final		
60	—	2.00	2.07	7.9 ± 0.05	
60	+	2.00	2.07	7.4 ± 0.02	
90	—	2.00	2.06	7.9 ± 0.10	14 ± 3
90	+	2.00	2.07	7.4 ± 0.05	17 ± 4

*Data are averages from three experiments followed by s.d.

†Efflux rate into HCl averaged over the 90-min period. Scutellum slices (0.5 g) were estimated to have a plasmalemma surface area of 0.048 m^2. This calculation is based on a cell diameter of 50 μm [20], and on the assumptions that 80% of the wt is inside the plasmalemma and that the cell contents have density of 1.0.

Chara indicate that the proton influx rate calculated for the scutellum cell at pH 2 is relatively low. In calculating this influx rate, it was assumed that sucrose–proton efflux balanced proton influx. This assumption is based on the idea that the two fluxes are connected through a transmembrane electrical potential, negative inside, which is set up by the sucrose gradient via sucrose–proton co-transport [16] (it is assumed that ATP-driven, electrogenic proton pumps in the plasmalemma do not function against the steep pH gradient present in the experiments of Table 2). If this is correct, proton influx is driven, in part, by sucrose–proton efflux, and an increase in the rate of one the fluxes should be followed by an increase in the rate of the other. When tetraphenylborate (TPB^-, a lipophilic anion) was added to the HCl solution at pH 2, the rate of sucrose efflux increased about 10-fold (unpublished results). Apparently, TPB^- acts as a porter in the plasmalemma, increasing the rate of proton influx [16, 24]. This result is entirely consistent with the above ideas.

Estimation of cytoplasmic pH using DMO

Since DNP increases glucose space and influences cytoplasmic pH (Table 1), the reliability of the pH estimates obtained with DNP was checked using DMO-^{14}C. Two experiments, each with five replicates, were run with DMO. Slices were incubated in buffered DMO (μM, pH 5.8) for up to 5 hr. DMO equilibrated between bathing solution and slices in 1–2 hr, and a constant distribution was maintained for at least 4 hr. DMO had no effect on glucose space which was 40 μl/0.5 g (cf. Table 1). DMO content of the vacuoles was calculated assuming the vacuoles had a pH of 5.4 (see above), and that 80% of the cell water was within the plasmalemma. From the DMO distributions between cytoplasm and bathing solution, an average cytoplasmic pH of 7.83 was calculated. The pH values of all replicates were within 0.05 unit of the average pH.

DISCUSSION

The assumptions made in using the distribution of DNP for estimating cytoplasmic pH are: (a) the protonated form of DNP readily penetrates into the cell, and at equilibrium, the concentration is the same inside and outside the cell, (b) the activity coefficient of DNP is equal throughout the system, i.e. the pK for DNP is the same inside and outside the cell, (c) DNP is not transported by an energy-linked system, (d) DNP does not precipitate or bind inside the cell and does not accumulate in spherosomes.

Except for (b) above, these assumptions are supported by the known behavior of DNP in cellular systems. The rapid, pH-dependent penetration of DNP into the slices (Figs. 1 and 3) indicates that the protonated form penetrates into the cells, and it is assumed that the equilibrium distribution represents the state where the concentration of the protonated form is the same inside and outside. The equilibrium distribution could also represent the state at which net efflux of the DNP anion is balanced by net influx of the protonated form. However, this is unlikely because the anion must exit with a compensating cation, e.g. K^+, and the net result would be an efflux of K^+ and an influx of protons. But at an outside pH of 5.5 or above DNP-induced proton influx was negligible (Fig. 2). The pK of DNP in the cytoplasm is not known, but the greatly increased concentration of the DNP anion in the cytoplasm (Table 1) may decrease the activity coefficient. This would cause an overestimate of cytoplasmic pH. The results with mannose-treated slices (Fig. 4) indicate that DNP is not transported by an energy-linked system, since mannose is an energy poison. DNP undoubtedly enters the spherosomes, most likely in the protonated form since the anion would require a lipid-soluble cation for accumulation. The partition coefficient for protonated DNP from 0.1 M KCl into decane is 2.2 in favor of the decane [25]. Using this value for the partition coefficient of DNP between spherosome and cytosol (pH 8), the calculated concentration of DNP in the spherosome is 1.2 μM, which would have a negligible effect on the cytoplasmic pH estimate (cf. Table 1).

In assessing the effect of external pH on cytoplasmic pH, it is assumed that the buffer (pH 6.1) used for pH measurement was not itself affecting cytoplasmic pH. Since it took only 20 min for DNP to equilibrate between bathing solution and slice (Fig. 3), it is unlikely that significant amounts of buffer ions, hydroxyl ions, or protons entered the cells during pH measurement. The constancy of the cytoplasmic pH estimates following incubation of slices for 1–2.5 hr at external pHs in the 2–8 range (Fig. 4, Table 2), the resistance of scutellar metabolism to HCl at pH 2, e.g. [15], and the lack of influence of external pH in the 4–8 range on sucrose synthesis from exogenous fructose [16] support this conclusion.

DMO is the weak acid most commonly used for pH measurement. One disadvantage of DMO is that it takes much longer to equilibrate than does DNP. In algae [2, 3] and higher plants [10], equilibration of DMO took 2–4 hr. Therefore, in studies on the effect of external pH on internal pH, the pH of the DMO-containing bathing solution was varied [3], which limits the pH range that can be tested. An advantage of DMO is that it appears not to affect ATP levels. In *Chara*, DNP (0.1 mM, pH 6) reduced the ATP level to 32% of the initial value, whereas DMO had no effect on ATP levels [26]. It is remarkable, therefore, that DMO and DNP gave very similar estimates of cytoplasmic pH in the scutellum.

A cytoplasmic pH of 8, the value determined here for the scutellum using DNP, is higher than that reported for most plant cells but similar to that reported for sieve tube contents [27]. The high pH may be caused by the abundance of mitochondria in the scutellum cell. The scutellum cell has a high respiratory rate [15] and functions primarily in transport. In cells with similar functions such as phloem companion cells and transfer cells, mitochondria make up as much as 20% of the cytoplasmic volume [28]. Presumably, the matrix of the mitochondrion has a higher pH than the cytosol because the electron transport system acts as a proton pump and sets up a pH and electrical potential difference across the inner membrane. The DNP distribution should reflect more closely the matrix (higher) pH than the cytosol (lower) pH [29].

DNP and mannose are both energy poisons. DNP

might not abolish the pH gradient across the inner membrane of the mitochondrion because the electron transport system will continue to pump protons, perhaps at even a greater rate. However, mannose acts differently; it almost completely inhibits the catabolism of endogenous sucrose [30], which is the major carbohydrate substrate in the scutellum, and it inhibits respiration about 50% (unpublished results). It is suggested, therefore, that when DNP is added to mannose-treated slices the pH difference between cytosol and mitochondrial matrix is abolished, and the DNP distribution reflects the pH of the cytosol. This suggestion is consistent with the observation that isolated, respiring mitochondria from various plants maintain a pH difference of 0.5 unit more alkaline than the solution bathing them [31]. The alternative suggestion, that mannose causes leakage of acids across the tonoplast, is not supported by the finding that increasing the incubation period in mannose from 1 to 2.5 hr did not further decrease the estimated cytoplasmic pH.

EXPERIMENTAL

Preparation of scutellum slices. Maize grains (*Zea mays* L. cv Funks 4949A) were soaked in running tap H_2O for 24 hr, then placed on moist paper towels and grown in the dark at 24–25° for 72 hr. Scutella were excised and cut transversely into slices of 0.5 mm or less in thickness (3–7 cells in thickness [20]). Slices were washed in H_2O until washings were clear, blotted on filter paper, and weighed into groups of 0.5 g.

Incubation conditions. Each group of slices was placed in a 50-ml beaker to which 10 ml of H_2O or experimental soln was added as described below. Solns and wash H_2O were removed from the beakers with a Pasteur pipette connected to a H_2O aspirator pump. Incubations were carried out on a water-bath at 30°. Slices were incubated in H_2O for 30 min, rinsed once with H_2O, and then incubated for 60 min in buffer ± mannose (50 mM). The buffers (20 mM) were the Na^+ salts of citric acid (pH 3, 4, 5), Mes (pH 6), Mops (pH 7) and Epps (pH 8). In some experiments, slices were rinsed with HCl (pH 2) at the end of the initial H_2O incubation and then placed in HCl^- mannose (50 mM, pH 2) and incubated for 60 or 90 min. At the end of the incubation in buffer, slices were rinsed with H_2O. At the end of the incubation in HCl, slices were rinsed with H_2O, with Mes (20 mM, pH 5.8), and finally with H_2O (the buffer rinse was included to remove protons from the cell walls). The beakers were wiped dry, and a filter paper wick was inserted under the pile of slices to remove the surface H_2O.

Measurement of cytoplasmic pH. Each group of washed slices from the buffer of HCl incubation was incubated in 10 ml of buffered DNP or DMO. When DNP was used, 0.1–0.3 ml of bathing soln were removed at intervals, mixed with Tris buffer (1.5 ml, 0.2 M, pH 9.0), made up to 4.0 ml, and the *A* measured at 360 nm. DNP content was calculated from the *A* 360 nm of DNP solns of known concn. When ^{14}C-DMO was used, 0.1-ml portions of the bathing solns were taken at the end of the incubation period and placed in vials. The slices were washed 2 × with 10 ml H_2O, 10 ml of 80% EtOH were added and boiled on a hot plate. After cooling, the vol. of the EtOH soln plus slices was adjusted to 35 ml with H_2O, and a 1 ml portion was placed in a vial. Slices incubated in ^{12}C-DMO were treated in the same way, and 1 ml portions of the EtOH extracts from these slices were placed in vials containing the 0.1-ml portions of ^{14}C-DMO bathing solns. In this way, both sets of vials (bathing soln and EtOH extract) had the same quenching characteristics. The ^{14}C content of the vials was measured by liquid scintillation counting. Cytoplasmic pH was calculated from the distributions of DNP and DMO between slices and bathing soln using the Henderson–Hasselbalch equation and p*K* values of 4.0 for DNP [32] and 6.3 for DMO [6]. The approximations and assumptions necessary for the calculation of cytoplasmic pH are discussed in the Results and Discussion sections.

Measurement of glucose space. It is necessary to know the cytoplasmic vol. in order to calculate cytoplasmic pH from DNP distribution. Scutellum cells have many small vacuoles, making it impossible to estimate cytoplasmic vol. from microscopic measurements. Therefore, the glucose space of the scutellum slices was used as a measure of cytoplasmic vol. Glucose space is defined as the vol. of H_2O necessary to contain the tissue glucose at the glucose concn of the bathing soln. Glucose space was measured by a modification of a method previously described [14]. The method is based on the observation that hexoses readily penetrate the scutellum plasmalemma and that hexose efflux is strongly inhibited by low temp. [14, 33].

Groups of slices were incubated in H_2O for 30 min, rinsed once, and placed in 10 ml of a buffered (20 mM) glucose (200 mM) soln ± DNP (0.1 mM). When DMO was used, slices were incubated in buffered DMO (μM) for 1 hr before placing them in buffered glucose (200 mM) plus DMO (μM). Slices were incubated in the glucose soln at 30° for 35 min (the time necessary for glucose to equilibrate between bathing soln and tissues [14]), and then beakers plus contents were placed in an ice-bath for 15 min. The chilled slices were rinsed once with ice-H_2O, 10 ml ice-H_2O were added and the beakers were replaced in the ice-bath for 11 min (the time necessary for glucose of the intercellular spaces to diffuse into the bathing soln [33]). The slices were filtered out on a Buchner funnel, rinsed once with ice-H_2O, transferred to a 100-ml beaker and killed with boiling 80% EtOH. The glucose content of the scutellum extracts was determined as previously described [34]. Glucose space in μl was calculated by the formula: μmol glucose × 10^3 μl/ml × ml/200 μmol.

Measurement of proton influx. In the experiments of Fig. 2, the beakers were removed from the water-bath at intervals, placed on a magnetic stirrer, and the pH of the bathing solns was adjusted to 3.5, 5.00 or 5.50 with 20 mM or 5 mM HCl. The bathing solns at pH 5 and 5.5 contained 0.1 mM citrate (pH 5.0) or Mes (pH 5.5) to stabilize the pH during pH adjustment.

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