The Effect of Reduced Water Potential on Soybean Mitochondria

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Abstract. The respiration of excised hypocotyls and of isolated hypocotyl mitochondria from soybean [Glycine max (L.) Merr., var. Wayne] was determined in various concentrations of sucrose and potassium chloride. Hypocotyl oxygen uptake declined with increasing solute concentration; no specific effects of either solute were apparent. Mitochondrial state III respiration was strongly inhibited as the solute concentrations were raised and there was in addition a specific inhibitory effect of the salt. State IV respiration, however, was unaffected by the presence of osmoticum. ADP/O ratios were also unaffected, except at high potassium chloride concentrations (470 mM). The primary effect of solutes was thus to limit the rate of substrate oxidation.

Hydrostatic pressure did not reverse the decline in net phosphorylation accompanying reduced oxidation. It was inferred therefore that the inhibition was not due to lower water potential per se, but rather to some other effect of water or solute concentration.

The effect of solutes on a mitochondrial enzyme, malate dehydrogenase, was also examined. Sucrose inhibited malate oxidation by both the mitochondria and the isolated enzyme in parallel, while potassium chloride was more inhibitory on the isolated enzyme. It was concluded that although the addition of solute lowers the water potential, the primary effects are exerted through specific effects of the solute on enzyme activity.

Although plant respiration is always retarded by the net loss of relatively large amounts of water, the effects of smaller changes in water content are variable. There are two main responses, either the respiration is stimulated with small decreases in water content or inhibition occurs at all water deficits (3,7).

The reasons for these changes in respiratory metabolism under conditions of stress have remained largely unexplored. Kursanov (15, see also 17) reasoning from the decrease in phosphorylated compounds reported by Zhokheevich and Koretskaya (24) and from the stimulated respiration that sometimes occurs during drought, concluded that oxidation and phosphorylation become uncoupled. Flowers (11) found potato disks to show an increasing inhibition of 14C-labeled acetate, glucose and succinate utilization, together with decreasing malonate sensitivity and vesiculation of mitochondrial cristae as the water potential was lowered. The results were taken to be indicative of mitochondrial malfunction under water stress.

Isolated mitochondria are affected by conditions of lowered water potential, generally increasing sucrose concentration (see 4). Plant mitochondria may show an increase in state IV respiration rate over a large range of solute concentrations (e.g. 0 to about 800 mM). Alternatively stimulation may be limited to the range from 0 to about 400 mM, when further increases in concentration either have no effect on or decrease the rate of oxidation. Animal mitochondria show a more uniform response with an optimal sucrose concentration of about 200 mM. State III respiration, however, is generally inhibited in both plant and animal mitochondria as the solute concentration is raised (14;12;21) although the degree of inhibition may vary with the particular substrate being oxidized (12,22).

The results obtained on the effects of low water potential on phosphorylation are also somewhat variable. Changes in concentration from about 0 to 500 mM in ADP/O ratios in 2 of Stoner's (22) experiments,
but had little effect in those of Johnson and Lardy (12) or Slater and Cleland (21). Cooper and Lehninger (5, 6), however, did report uncoupling by sucrose in digitonin fragments of rat liver mitochondria.

The mitochondrial system was an obvious choice for the further investigation of the effects of water deficit on plant respiration. However, since water potentials are most commonly lowered osmotically, evaluation of the data requires recognition of specific solute effects and evaluation in distinction to water potential. Experiments with these aims are reported here.

Materials and Methods

Seedlings of soybean [Glycine max (L.) Merr., var. Wayne] were grown at 29° in the dark for approximately 88 hr. Seeds were normally planted in plastic trays containing about 450 g of vermiculite and watered with 2.2 l of 0.1 M calcium chloride solution. 1.7 l on planting and 0.5 l after 40 hr growth. The surface of the vermiculite was lightly dusted with “Spargan” bulb dust (Science Products. Chicago, Illinois) and the trays covered with aluminum foil. Seedlings were raised at lower water potentials by raising the ratio of vermiculite to calcium chloride solution.

A modification of the procedure of Kenefick and Hanson (13) was used to isolate mitochondria. About 100 g of excised hypocotyls were washed with distilled water (3 times with 500 ml at room temperature and 3 times with ice cold water) before grinding in an icecold mortar with 250 ml of a cold isolation medium consisting of sucrose (400 mM); TESa (50 mM, pH 7.8) cysteine (2 mM); EDTA (5 mM) and BSA (1 mg ml⁻¹). The homogenate was filtered through cheese cloth and centrifuged at 500g at 0° for 10 min. After transfer of the supernatant to clean tubes the mitochondria were sedimented at 15,400g for 10 min and the pellets then resuspended in 50 ml of cold 400 mM sucrose. Following a further centrifugation at 500g the supernatant was again transferred to clean tubes and 10 ml of cold 600 mM sucrose layered below each suspension. Finally after centrifugation at 9800g for 20 min the supernatant was discarded and the final suspension made in 2 to 3 ml of 400 mM sucrose.

A Clark oxygen electrode was used in conjunction with a Gilson oxygraph to measure oxygen uptake. Mitochondria (0.5-0.8 mg protein) were suspended in 3 ml of a reaction mixture consisting of BSA (1 mg ml⁻¹); TES (20 mM, pH 7.4); malate (10 mM); NAD (0.38 mM); CoA (0.13 mM); succinate (10 mM) and phosphate (3.8 mM) together with an osmoticum. Succinate (10 mM) was used in place of malate in a number of assays as indicated in the text. The pH was adjusted with KOH. The oxygen content of reaction mixtures was determined using protocatechuic 4-5-oxygenase with protocatechuic acid as substrate; 1 mole of protocatechuic acid is equivalent to 1 mole of O₂ (cf. Dagley et al. 8). All measurements were made at room temperature (23° ± 1°). Acceptor control ratios (ACR) and ADP/O ratios were determined after the addition of 200 to 350 nmols of ADP, as described by Estabrook (10).

Oxygen uptake of hypocotyls was determined at 25° by standard Warburg manometry. Six 2-cm hypocotyl segments (approximately 0.4 g fresh wt) were placed in each flask and O₂ measured over a period of 1 hr. Hypocotyls grown under normal conditions were used for experiments involving osmotically induced water deficits and in these experiments the osmoticum was buffered with TES (50 mM, pH 7.5). The respiration rates of hypocotyls from seedlings raised under drier conditions were determined in dry flasks.

Both mitochondrial and hypocotyl protein was estimated using the method of Lowry et al. (16) with BSA as standard. Dried hypocotyls were extracted once with trichloroacetic acid (5 % w/v) before protein was dissolved overnight (at 40°) in N-NaOH.

Phosphorylation was determined in the pressure experiments (see below) by estimating the change in P₁ in a medium consisting of BSA (1 mg ml⁻¹); TES (20 mM, pH 7.4); pyruvate (10 mM); malate (10 mM); NAD (0.38 mM); TTP (0.3 mM); CoA (0.13 mM); MgCl₂ (1.3 mM); Pi (2.0 mM); hexokinase-Sigma Type III-(0.3 mg ml⁻¹); glucose (50 mM); ADP (1.6 mM); osmoticum and 0.3 or 0.8 mg of mitochondrial protein. The inorganic phosphate was measured in supernatants containing 5 % (w/v) cold trichloroacetic acid following the method of Pennill (20). Phosphorylation reactions were carried out in 5 ml syringes and where pressure was applied this was achieved within 1 min of adding the mitochondria, by applying weights to a triple beam balance supporting the plunger of the syringe on the pan. The reaction was not allowed to exhaust O₂ in the solution, as determined previously with the oxygraph.

Malate dehydrogenase (1.1.1.37.) was prepared by sonicating (Biosonik. Model BP1—Blackstone Corporation. Jamestown. New York) freshly isolated mitochondria in 400 mM sucrose for approximately 2 minutes at 0°. The sonicated preparation was then centrifuged at 27,000g for 5 min and the supernatant used as enzyme, following a 250 fold dilution. Enzyme activity was assayed at room temperature according to the procedure of Ochoa (19). Changes in optical density at 340 nm were recorded with a GPO electronic spectrophotometer.

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*The following abbreviations are used in the text: TES for N-tris (hydroxymethyl)-methyl-2-aminoethanesulphonic acid; BSA for bovine serum albumin; TTP for thiamine pyrophosphate; CoA for coenzyme A.
Water potentials\(^4\) and osmotic potentials were measured with the thermocouple psychrometers of Boyer and Knipling (2).

**Results**

**Hypocotyl Respiration.** The uptake of O\(_2\) by hypocotyl segments was retarded by lowering their water potential osmotically (Fig. 1). Tissue water potentials were estimated after correcting the solution water potential for the amount of water lost from the tissue. There were no apparent differences between the effects of potassium chloride and of sucrose and from the combined results an inhibition of 1.2% per bar was calculated.

Although water deficits imposed on the seedlings during the 3.5 day growth period had a marked effect on hypocotyl length and weight (lowering the hypocotyl water potential from \(-2.2\) bars to \(-4.0\) bars reduced the mean weight of a single hypocotyl from 0.29 g to 0.09 g and the mean length from \(77 \pm 4\) mm to \(24 \pm 2\) mm) there was no significant reduction of the respiration rate. The constancy of respiration rate was correlated with a relatively unchanged water potential and/or water content.

**Mitochondria.** The hypocotyls used for the isolation of mitochondria had an average water potential of \(-1.94 \pm 0.06\) bars. The osmotic component of the total potential was \(-5.24 \pm 0.02\) bars, giving an estimated turgor pressure of +3.3 bars. The isolation was carried out under hypertonic conditions at an osmotic potential of \(-13.8\) bars and the mitochondria remained at \(-11.0\) bars in a 400 mM sucrose solution at 0\(^\circ\)C until used. Storage did not normally exceed 2 hr: acceptor control ratios measured in 230 mM sucrose did not decrease in this time.

Addition of ADP to mitochondria oxidizing malate+pyruvate caused a normal state IV to state III transition (Fig. 2A). The return to state IV respiration was followed after approximately 1 min by a spontaneous increment in the respiration rate (see Fig. 2). This increment was apparent in all sucrose concentrations used (up to 930 mM), although somewhat reduced in magnitude at the higher concentrations. In KCl solutions the increment was not apparent at concentrations greater than 230 mM. Since the nature of this stimulated respiration was not understood, only the results obtained with the initial state IV rate will be reported: acceptor control ratios and ADP/O ratios were calculated from this initial state IV rate.

Succinate+pyruvate was oxidized at high rates, particularly during state IV respiration (Fig. 2B). Acceptor control ratios were consequently lower than with malate+pyruvate. There was no spontaneous increment in the state IV rate with succinate. Addition of KCl or sucrose to lower the water potential did not alter the relative rates of succinate and

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\(^4\) Water potential (\(\Psi_w\)) is defined by, 
\[
\Psi_w = \mu_w - \mu_w^0 \frac{V_w}{V_w^0}
\]
where \(\mu_w^0\) and \(V_w^0\) are the chemical potential and partial molal volume respectively of pure free water at atmospheric pressure and a given temperature and \(\mu_w\) is the chemical potential of water in the system under consideration. The value of \(\mu_w\) is a function of the number of insoluble substances (matric potential, \(\Psi_T\)), soluble substances (osmotic potential, \(\Psi_w\)), and pressure (\(\Psi_p\)) such that 
\[
\Psi_w = \Psi_T + \Psi_w + \Psi_p
\]
malate oxidation (cf. 12 & 22): state III rates were 1.3 times higher and state IV rates twice as high in succinate+pyruvate as in malate+pyruvate at all concentrations of solute. Only data obtained with malate+pyruvate are reported below.

Acceptoless (state IV) respiration was only slightly influenced as the concentration of osmoticum was raised (Fig. 3): maximum rates occurred in solutions with an osmotic potential of about −10 bars. Rates in KCl and in sucrose solutions were similar.

Fig. 3. Oxygen uptake by mitochondria at various water potentials. Solid lines, state III rates in KCl (○) and sucrose (●) solutions; broken lines, state IV. Molarities of sucrose used were 0.23, 0.47, and 0.93 and of KCl, 0.12, 0.27, and 0.47.

State III respiration rates were strongly inhibited as the water potential was decreased. Although not shown, rates in the absence of added solute were occasionally lower than with 120 mM KCl or 230 mM sucrose. The inhibition of state III respiration was more marked than with intact tissue (cf. Fig. 1): hypocotyl respiration was inhibited by 1.1% per bar while mitochondrial respiration was inhibited by 4.9% per bar by sucrose. In addition there was a pronounced effect of salt over and above that of sucrose (inhibition by KCl was 8.9% per bar). Experiments with alternative solutes indicated that the effects of glucose were similar to those of sucrose and NaCl to KCl.

Since state IV respiration was largely unaffected by increased solute concentration, the marked decline in ACR (Fig. 4) occurring with decreased osmotic potential was due to the decline in state III respiration.

Although the ACR declined as the osmotic potential was lowered this was not the case for ADP/O ratios. Neither sucrose nor KCl had any significant effect upon the latter (Fig. 4).

Fig. 4. Acceptor control ratios at various water potentials in KCl (○) and sucrose (●) solutions. The mean values from a number of experiments are plotted: there were no significant differences between ratios calculated from a first or a second addition of ADP. Standard errors were less than 1%.

(Fig. 5). Below −15 bars, however, lowering the water potential was accompanied by some decline in the ADP/O ratio, particularly in KCl. It is clear that the primary effect of a lowered water potential on soybean mitochondria was not uncoupling of the respiration from phosphorylation. Evidently the inhibitory effects of increasing solute are only observed
Table I. Phosphate Esterification at Various Water Potentials

Mitochondrial protein (0.3 mg) was incubated for approximately 10 min in the phosphorylation medium described. The water potential was lowered by the addition of KCl and then adjusted with hydrostatic pressure as indicated.

<table>
<thead>
<tr>
<th>Osmotic potential of assay medium (bars)</th>
<th>Phosphorylation at pressure of 0 bars (nmoles per mg protein per min)</th>
<th>+9.2 bars</th>
<th>+11.5 bars</th>
</tr>
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<tbody>
<tr>
<td>- 3.3</td>
<td>849</td>
<td>836</td>
<td>889</td>
</tr>
<tr>
<td>- 8.5</td>
<td>840</td>
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</tr>
<tr>
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<td>847</td>
</tr>
<tr>
<td>-23.6</td>
<td>106</td>
<td>106</td>
<td>847</td>
</tr>
</tbody>
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with high respiration rates. The limitations imposed on oxidation must be through effects on substrate penetration, electron transport or the coupled phosphorylation mechanism.

At this point it became important to distinguish between effects of water potential and those due to solute concentration. If the noted decline in respiration with increased solute were due to the lowered water potential, it should be possible to reverse the decline by raising the potential with pressure. Technical difficulties prevented following respiration under pressure. The procedure adopted for this experiment (determination of P1) made use of the fact that total phosphorylation reflected respiration (since ADP/O ratios were not significantly changed). A hexokinase trap was added to the medium and the reaction run in a hypodermic syringe which could be loaded with weights to give a known pressure. The experiment was terminated prior to the exhaustion of oxygen in the solution as determined by separate measurement with the oxygen electrode.

At no time could an effect of pressure on net phosphorylation be detected (Table I). Hence there is no evidence that water potential per se affects mitochondrial activity. The strong inhibition must be due to increasing solute concentration.

Malate Dehydrogenase. Further indication that the effect of solute was due to a specific effect on reaction rates was obtained by assays of malate dehydrogenase activity at various solute concentrations. The enzyme was markedly inhibited by both sodium chloride and potassium chloride solutions (Fig. 6): rates in 470 mM solutions were 10% of the rate in water. Sucrose also retarded the rate, but not as severely as the salt solutions: the rate in 930 mM sucrose was still 51% of the control rate. A noticeable feature of the inhibition in sucrose was that the relative inhibition of malate dehydrogenase activity corresponded closely to the retardation of O2 uptake (Fig. 7) by mitochondria oxidizing malate. This relationship was less obvious with potassium chloride as the solute. At low concentrations of KCl (120 mM) malate dehydrogenase activity was greatly reduced although O2 uptake was hardly affected. With concentrations greater than 120 mM however, unit decrease of malate dehydrogenase activity was more nearly associated with unit decline of O2 uptake.

The explanation may lie in penetration of the mitochondria by potassium chloride. In the absence of substrate mitochondria swelled spontaneously when reminerized from sucrose to KCl solutions, presum-
ably due to the penetration of salt. With substrate present, however, only a small change in optical density (520 mm) was apparent. It was envisaged that at low salt concentrations, little or no KCl penetrated, so that there was little inhibition of dehydrogenase activity. As concentrations rose, however, greater salt penetration caused greater retardation of enzyme activity. The displacement of the KCl curve from the 45° angle would have its explanation in the ability of the mitochondria to exclude most of the salt from the internal enzyme. The internal concentration of salt would be less than the external. If the sucrose effect were in some way due to a change in water content of the mitochondria, then it is probable that the effect of the salt was due to both osmotic and specific effects. The enzyme active site (or at least the site of action of potassium chloride) would have to lie within the inner membrane, the presumed site of KCl exclusion, in the presence of substrate.

Discussion

Increasing solute concentrations clearly depressed both hypocotyl and mitochondrial state III respiration, being several fold more effective on the latter. The addition of solute has several effects which may be responsible for this result: declining water potential, osmotic withdrawal of water and/or increasing reaction of the solute.

Lowering the water potential should depress any reaction in which water is a reactant. Dixon and Webb (9) attribute the inhibitory effects of high sucrose concentration in the experiments of Nelson and Schubert (18) with β-fructofuranosidase to lowered water activity. However, in our experiments, hydrostatic pressure—an important factor in the water activity in turgid plant cells—did not alter net phosphorylation in the presence or absence of osmoticum (table 1). Thus we have no evidence that water potential as such plays any part in the solute inhibition of respiration. Equally convincing is the sucrose inhibition of malate dehydrogenase (Fig. 6) which catalyzes a reaction where water is not a reactant, at least in the formal chemical sense.

The effect of changing water potential on osmotic systems must also be considered. Since plant mitochondria show extensive water removal and condensation of the matrix in 400 mM sucrose (23), osmotic water loss might concentrate certain endogenous solutes to the inhibitory level or introduce physical limitations manifested in lowering of substrate diffusion rates as proposed by Johnson and Lardy (12). In our experiments, however, malate oxidation by mitochondria and malate dehydrogenase activity from sonicated mitochondria showed parallel sucrose inhibition (Fig. 7). If there were any physical barriers they were not unique to the osmotically contracted mitochondrion but to the enzyme itself.

This analysis suggests that the critical response to increasing solute lies with one or more enzymes. Either the solute acts directly on the enzyme or does so indirectly by altering some aspect of enzyme hydration. In the case of the inhibition of malate dehydrogenase (Fig. 7) the steady decline in activity with increasing sucrose concentration might be attributed to gradual changes of water structure surrounding the enzyme (cf. 14). This may result from specific interactions between water and solute. However, potassium chloride clearly produces in addition some drastic change in the properties of the enzyme.

The absence of specific effects of either solute on the hypocotyl respiration (Fig. 1) probably reflects the ability of the plasmalemma to restrict the penetration of KCl as compared with the mitochondrial membrane. The different sensitivities of tissue and mitochondria may have resulted from the unlikelihood that, in vivo, all of the mitochondria are respiring at state III rates, where the solutes are inhibitory.

As we noted in the introduction, it has been proposed that lowered water content (or potential) decreases phosphorylative efficiency. This generalization is definitely not true of soybean mitochondria, except possibly at high solute concentrations. It may prove relevant that hypocotyl respiration is not stimulated in this tissue by water deficit. Net phosphorylation is of course reduced concomitant with the fall in respiration.

Although the water potential concept is extremely useful in problems of water flux we cannot see the absolute relevance to the effects of water deficit on enzyme activity. The concept has its value, however, in expressing the extent of water withdrawal from osmotic systems where metabolic functions are being determined.

Acknowledgments

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Literature Cited