Use of ^{31}P NMR to Assess Effects of DNP on ATP Levels *in Vivo* in Barley Roots

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ABSTRACT

Previous work has shown that undissociated 2,4-dinitrophenol (DNP) both increases the permeability of roots to ions and alters the membrane lipids of barley roots. Anionic DNP is the main entrant form but has no effect on permeability or on the membrane lipids. The amount of anionic DNP taken up by the roots is sufficient, that were it in free solution in the cytoplasm, the DNP would uncouple oxidative phosphorylation, and thereby inhibit ATP synthesis. The present work was undertaken to assess whether DNP alters ATP levels when it is taken up by barley roots. ^{31}P nuclear magnetic resonance spectra were used to monitor, *in vivo*, levels of ATP, cytoplasmic phosphate, vacuolar phosphate, and other phosphate compounds in barley roots in the presence of 10 micromolar DNP at pH 5 and pH 7. The spectra indicate that no change in the level of ATP or the cytoplasmic pH occurred in the roots in the presence of DNP for as long as 20 hours. Thus, the effects of undissociated DNP are effects directly on the root membranes and do not involve inhibition of ATP synthesis. Furthermore, the results explain why anionic DNP has no effect on ion uptake and accumulation.

The protonated (undissociated) form of DNP[1] increases the permeability of barley roots to ions (4) and simultaneously alters the membrane lipid composition (6). Anionic DNP is the main entrant form but has no effect on either ion uptake and permeability or the membrane lipids. The amount of anionic DNP taken up by the roots (0.1 to 1 mmol/L cell water) is sufficient to inhibit mitochondrial ATP synthesis were it largely in solution in the cytoplasm. However, such inhibition might be prevented in an intact tissue by: (a) a concentration of phosphate in the cytoplasm sufficient to compete with the action of DNP (1, 5) or (b) if most of the DNP became inaccessibly bound (2). Consequently, we have undertaken a study to assess whether DNP inhibits ATP synthesis in barley roots *in vivo*. This was done by monitoring, *in vivo*, the levels of ATP, G6P, Cyt Pi, Vac Pi and the cytoplasmic and vacuolar pH in barley roots by the use of ^{31}P NMR.

MATERIALS AND METHODS

Plant tissue for the experiments was roots of 6 d old seedlings of barley (*Hordeum vulgare* L. cv Compana) which had been grown in aerated 0.1 mM $CaSO_4$ at pH 5.6 in the dark at 25°C. For each experiment, about 400 root tips, 1 cm long, were excised and rinsed in 0.1 mM $CaSO_4$ just before use. At the start of the experiment, a suspension of root tips in 0.1 mM $CaSO_4$ was placed in a 10.0 mm NMR tube. The roots in the probe were perfused continuously with 1 L of aerated 0.1 mM $CaSO_4$ without or with DNP at the rate of 45 ml/min. The perfusion system (10) is a modification of the system described by Lee and Ratcliffe (7). The solution perfused through a capillary tube into the NMR probe without turbulence. The pH of the perfusing solution was monitored during treatment and maintained by periodic titration with solutions of KOH or HCl. Treatments were changed by replacement of the perfusion solution. All treatment solutions contained 0.1 mM $CaSO_4$ and were aerated continuously.

The ^{31}P NMR experiments were performed at 161.7 MHz with a 54 mm narrow bore spectrometer at 21 to 23 °C. Each spectrum required 10,000 transients with a repetition time of 0.162 and a 30° pulse. The total time of accumulation was 27 min per spectrum. Hexamethylphosphoramide was used as a reference standard for all ^{31}P spectra (10). An aqueous solution of 0.120 M HMPA in a 1.8 mm capillary tube was placed in the center of the NMR tube. Concentrations of NMR-visible phosphorus compounds in the roots (Table II) were determined from the spectra shown in the figures by comparisons of the areas of ^{31}P resonance shown by the roots with the area of HMPA resonance. The HMPA resonance was calibrated by comparison of the HMPA area of resonance with areas of resonance from 1 mM standard solutions of ATP, G6P, and Pi. Spectra of both the root tissue and the standard solution were obtained under quantitatively comparable conditions; *i.e.* 90° pulses and slow 20-s repetition rates. Adjustments for variations in signal response were made to establish a direct relationship between slowly and rapidly acquired spectra. The concentrations are expressed on the basis of the volume in the NMR tube (3.6 ml) and represent minimal concentrations. This was done because the roots occupy 45 to 50% of the volume of the tube and the cytoplasmic and vacuolar volumes are unknown.

Intracellular pH was estimated by use of a standard reference curve of pH as a function of chemical shift, which was obtained according to the method of Roberts *et al.* (11). In general, the pH values obtained throughout our studies are consistently 0.2 to 0.3 pH units higher than those reported by Roberts *et al.* (11). The somewhat higher values may be a result of faster perfusion rates in our studies (45 ml/min) or they may be a function of different susceptibility contributions of different magnet geometries.

RESULTS

NMR spectra of roots in aerated 0.1 mM $CaSO_4$ (control) show well-defined peaks of resonance for G6P; Cyt Pi; Vac Pi; γ-, α-,

[1] DNP, 2,4-dinitrophenol; NMR, nuclear magnetic resonance; G6P, glucose-6-phosphate; Cyt Pi, cytoplasmic phosphate; Vac Pi, vacuolar phosphate; HMPA, hexamethylphosphoramide; UDPG, uridine diphosphoglucose; MDP, methylenediphosphoric acid.

and β-ATP; and β- and γ-UDPG (with contribution of some NAD to the β peak of UDPG) in Figure 1A. Each spectrum is the average of 10,000 scans and takes 27 min to accumulate. The assignment of the labeled peaks is according to the method of Roberts *et al.* (11). Calibration for the pH dependence of the chemical shift for the resonance of Cyt P_i and Vac P_i indicates that the cytoplasmic pH is 7.6 and the vacuolar pH is 5.5. Continued maintenance of the roots in aerated 0.1 mM $CaSO_4$ for several hours (Figs. 2A and 3A) did not alter the spectra, or the pH of the cytoplasm and vacuole appreciably (Table I).

We estimated the concentrations of ATP, Cyt Pi, and Vac Pi shown in Table II from spectra shown in the figures. Concentrations of these compounds increased slightly over 3 h of perfusion with 0.1 mM $CaSO_4$ and then remained constant.

Perfusion of the roots with aerated 10 μM DNP at pH 7 for 3 h likewise did not alter the spectrum. And no change was evident when subsequently the roots were perfused for 4 h with 10 μM DNP at pH 5 (Fig. 1B). The concentrations of ATP (Table II) did not decrease over the course of 7 h of exposure of the roots to 10 μM DNP (3 h at pH 7 followed by 4 h at pH 5). A slight increase in the intensity of Vac Pi resonance, which was accompanied by a narrowing of the peak, corresponds to only a 5% increase in Vac Pi concentration. Values of the cytoplasmic and vacuolar pH likewise remained essentially constant (Table I). DNP has no consistent or appreciable effect on the cytoplasmic pH. Furthermore, the spectra and the cytoplasmic and vacuolar pH remained unchanged at 26 h in 10 μM DNP (Table I).

The roots were treated with 2-deoxyglucose on the premise that the high concentration of Cty Pi might compete with the action of DNP (1, 5). Figure 2B shows that 1 mM 2-deoxyglucose in 1.5 h markedly decreased Cty Pi and ATP concomitantly with a large increase in resonance at the position of G6P and increase in the vicinity of UDPG. Presumably, the increases are due to formation of 2-deoxy derivatives of G6P and UDPG. The 2-deoxyglucose had little or no effect on Vac Pi. Upon removal of

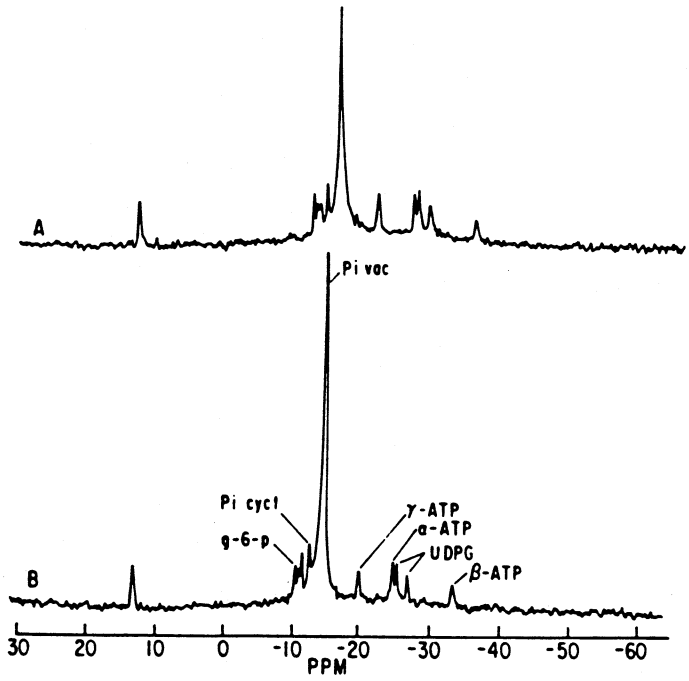

FIG. 1. NMR spectra of barley roots treated with DNP. A. Control roots in 0.1 mM $CaSO_4$ for 0.5 h. B. Roots in 10 μM DNP +0.1 mM $CaSO_4$ (pH 7) for 3 h, then perfused with 10 μM DNP + 0.1 mM $CaSO_4$ (pH 5) for 3.5 h. Spectra were obtained over a period of 27 min (10,000 transients) with a 30° pulse angle, a recycle time of 0.162 s and 2000 data sampling points zero filled to 16,000.

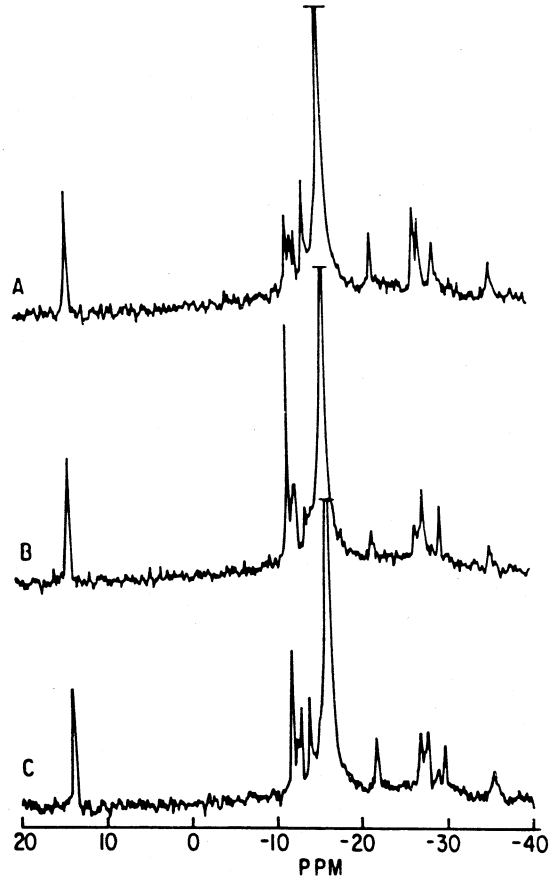

FIG. 2. NMR spectra of barley roots treated with 2-deoxyglucose followed by DNP. Description of the parameters for the NMR spectrum are given in the legend of Figure 1. The peaks are identified as shown in Figure 1B. The spectra have been expanded vertically to accentuate the Cyt Pi and ATP resonances. A. Control roots in 0.1 mM $CaSO_4$ for 1 h. B. Roots in 1 mM 2-deoxyglcuose + 0.1 mM $CaSO_4$ for 1.5 h. The peaks in the positions of G6P and UDPG contain the 2-deoxy derivatives. C. Roots transferred from the 2-deoxyglucose solution to 10 μM DNP + 0.1 mM $CaSO_4$, pH 7 for 4 h.

the 2-deoxyglucose and perfusion with an aerated solution of 10μM DNP at pH 6.6 for 3 h, no further decrease in ATP or alteration of the cytoplasmic pH ensued. Rather, the nucleotides and Cyt Pi increased and the 2-deoxy-derivatives decreased (Fig. 2C, Table II). The trend continued over 17 h in the DNP solution (Table I).

The roots were treated with a 10× higher concentration of DNP so that a greater proportion of DNP would be accessible for inhibition of ATP synthesis. Roots were perfused with 0.1 mM DNP, first at pH 7 for 2 h. At this pH, permeability increases and membrane lipid changes caused by protonated DNP would be expected to be minimal (4, 6). The result was no apparent change in the NMR spectra. When the DNP solution was changed to pH 6, a condition under which the 10× higher concentration of protonated DNP causes the roots to lose endogenous and accumulated salts as well as substrates, resulting in respiratory inhibition (4), the spectra at 3.5 h showed slight losses of nucleotides (a 22% decrease in ATP) and G6P but no alteration of the cytoplasmic pH (Fig. 3B, Table I). Vac Pi was unaffected under these conditions.

DISCUSSION

The uptake of DNP by barley root tips would be expected to affect the NMR spectra markedly. Oxidative phosphorylation by

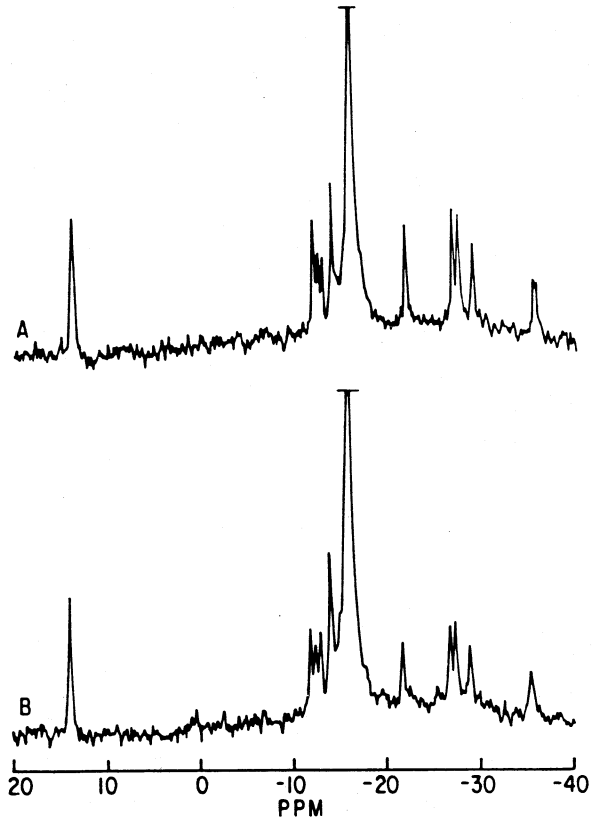

FIG. 3. NMR spectra of barley roots in 0.1 mM DNP. Description of the parameters for the NMR spectrum are given in the legend of Figure 1. The peaks are identified as shown in Figure 1B. A. Control roots in 0.1 mM $CaSO_4$ for 3 h. B. Roots in 0.1 mM DNP + 0.1 mM $CaSO_4$ (pH 7) for 2 h, the transferred to 0.1 mM DNP + 0.1 mM $CaSO_4$ (pH 6) for 3.5 h.

isolated barley root mitochondria was inhibited 75 to 80% by 10 μM DNP (5). DNP, as an uncoupler of oxidative phosphorylation, is known to dissipate proton gradients across membranes (3, 9). Diminution of the ATP level and alteration of the cytoplasmic pH would be the expected result, particularly inasmuch as ATP synthesis is rate-limiting in the barley roots (5). However, neither anionic nor protonated DNP had an appreciable effect on either ATP levels or the cytoplasmic pH. This is shown by the constancy of the ATP levels and pH of roots in the 10 μM DNP solutions, whatever the pH (Figs. 1 and 2, Tables I and II). The concentration of protonated DNP increases by a factor of 90 from pH 7 to pH 5 where it is approximately 10% of the total DNP in solution.

Table II shows that Cyt Pi concentrations of controls varied from 0.93 to 1.22 mM. Concentrations in roots treated with either 10 μM or 0.1 mM DNP at pH 7 are 1.11 and 1.13 mM. These values are within the range of control values and, therefore, are not significantly different. The concentrations in roots treated with 10 μM DNP at pH 5 or 0.1 mM DNP at pH 6 are 1.27 and 1.3 mM. These values are 13 to 17% higher than the values for the other 2 DNP treatments, which do not differ significantly from control concentrations. Thus, the 1.27 and 1.3 mM concentrations are of questionable significant difference. If real, they do not support uncoupling action of DNP since ATP did not decrease appreciably. Rather, the two higher concentrations seem to reflect the concentration of protonated DNP which is 0.8 μM in 10 μM DNP at pH 5 and 0.9 μM in 0.1 mM DNP at pH 6. The higher Cyt Pi concentrations may be a consequence of the permeability increase, such as lysis of phosphates that are normally invisible to NMR.

Table I. *Constancy of Cytoplasmic and Vacuolar pH of Roots*

Roots in the NMR tube were perfused constantly with the aerated treatment solutions. The pH was calculated from the chemical shifts of the respective peak resonances. δ ppm represents the ppm relative to external HMPA in a reference capillary whose shift is given as 13.78 ppm in reference to MDP (11). A shift position of −12.00 ppm for 2-deoxy-G6P corresponds to a cytoplasmic pH of 7.5. The insensitivity of the shift change to the pH of Pi at −16.00 ppm of the pH profile causes difficulty in the assessment of small changes in the pH of the vacuoles.

Treatment	2-Deoxy-G6P	Cytoplasmic pH	Vacuolar pH
		δ ppm	
Control (0.1 mM $CaSO_4$)			
1 h		7.6 (−13.96)	5.5 (−16.06)
3 h		7.6 (−13.92)	5.5 (−16.02)
23 h		7.5 (−13.98)	
+ 10 μM DNP			
pH 7, 3 h		7.4 (−14.07)	
pH 5, 4 h		7.6 (−13.96)	5.5 (−15.97)
26 h		7.5 (−13.99)	5.5 (−16.06)
+ 0.1 mM DNP			
pH 7, 2 h		7.6 (−13.90)	
pH 6, 4 h		7.5 (−14.03)	
21 h		7.4 (−14.07)	5.5 (−16.02)
+ 1 mM 2-deoxyglucose			
1.5 h	(−12.00)	7.5	5.5 (16.10)
↳ 10 μM DNP			
pH 7, 5 h	(−11.90)	7.6	5.5 (15.99)
17 h	(−11.89)	7.6	5.5 (16.01)

Table II. *Effect of DNP on Relative Content of Phosphate and ATP*

Quantitative data were obtained from the respective resonances by area comparisons with the standardized HMPA reference peak. Standard deviations are within ±9% for all values.

Treatment	Cyt Pi	Vac Pi	ATP
		mM	
Control (0.1 mM $CaSO_4$)			
1 h	1.03	15.0	0.44
3 h	1.22	15.2	0.43
Control 1 h	0.93	16.2	0.67
+10 μM DNP			
pH 7, 3 h	1.11	15.1	0.73
pH 5, 4 h	1.30	15.3	0.69
Control 3 h	1.10	15.0	0.41
+0.1 mM DNP			
pH 7, 2 h	1.13	15.1	0.43
pH 6, 4 h	1.27	15.3	0.32
Control 1 h	1.05	15.0	0.38
+1 mM 2-deoxyglucose			
1.5 h	0.67	15.3	0.11
↳ 10 μM DNP			
pH 7, 4 h	0.63	15.4	0.34

Decrease in G6P does not support uncoupling action of DNP either, in these experiments. G6P could decrease by uncoupling action only if ATP decreases, which it does not. G6P in control roots decreases over 20 h which probably results from a glucose limitation. This and the degree of respiratory decrease over this period, shown in previous work (4) indicate that substrate becomes more limiting.

As stated before, the ATP concentration did not decrease in roots treated with 10 μM DNP at either pH. It decreased slightly (22%) in roots treated with 0.1 mM DNP for 4 h at pH 6 (Fig.

3), but whether this change is significant is questionable. If real, how much this reflects the permeability increase must be considered. The concentration of protonated DNP under these conditions is sufficient to cause a nearly total loss of ions and a loss of substrates with resultant respiratory inhibition (4). Thus, the slight decrease in ATP, if real, is likely to be a result of the permeability increase rather than a result of inhibition of phosphorylation. ATP did not decrease in 4 of the 5 treatments. In fact, it increased upon exposure of the roots to DNP after treatment with 2-deoxyglucose. Thus, DNP did not cause any consistent or appreciable decrease in ATP concentration. Furthermore, the constancy of the intracellular pH (Table I) is consistent with the lack of any apprecable effect on ATP synthesis.

Our results contrast greatly with those reported by Kime *et al.* (8) who observed losses of ATP, UDPG, and G6P and collapse of the pH gradient in root tips of maize seedlings treated with 0.5 mM DNP at pH 6 for 6.5 h. The large increase in permeability which is likely to result from such a high concentration of protonated DNP (5 μM at pH 6) causes great difficulty in the assessment of whether the DNP effects are a direct result of uncoupling, *per se*, or a result of the permeability increase, or a combination of both. Because of this, we did not test concentrations of DNP higher than 0.1 mM. An important corollary to this is that the alteration of membrane permeability and lipid composition in the absence of inhibition of ATP synthesis indicates that protonated DNP affects the membranes directly.

Several possibilities for the failure of DNP to decrease ATP in barley roots *in vivo* need to be considered. The explanation must lie in the complexities of the tissue because DNP markedly inhibits ATP synthesis by mitochondria isolated from barley roots at 10 μM (5), a concentration used in the present study. Among the various possibilities, insufficient uptake of DNP is not likely to be a factor. DNP uptake is greatest at pH 6 or higher (4). The uptake from 10 μM DNP is sufficient to produce an internal concentration of 0.3 mM in 3 h and of 1 mM in 24 h, expressed on the basis of cell-water volume. In the present work, the deep yellow of the root tips in DNP solutions indicates substantial uptake of DNP. Previously, the intensity of the yellow stain was correlated with DNP uptake (PC Jackson, unpublished data).

Evidently, the concentration of Cyt Pi also is not likely to account for the failure of DNP to alter ATP levels. Markedly decreasing the level of Cyt Pi by treatment with 2-deoxyglucose did not produce an effect although this would be expected. The 2-deoxyglucose also decreased the level of ATP. Since ATP synthesis is rate-limiting in barley roots (5), Cyt Pi could not be depleted by longer exposure to 2-deoxyglucose without depleting ATP. That 2-deoxyglucose did not alter Vac Pi appreciably indicates the discreteness of compartments within the barley root tissue.

The most likely explanation for the failure of DNP to alter ATP levels is that intracellular amounts of free DNP in solution in the cytoplasm of the roots are not apt to be appreciable. This has been discussed before in detail (4) and will not be repeated here except to mention that no free phenol could be found in barley roots that had taken up hydroquinone (2). All of the phenol accumulated was associated with a glycoside. Such binding of DNP in an intact system is consistent with the ability of DNP to uncouple mitochondria isolated from barley roots (5) where phenolic binding is likely to be minimal. These results emphasize the difficulty in the assessment of inhibitor actions in an intact tissue.

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