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The energetic state of the thermogenic appendix of the voodoo lily inflorescence. A ^{31}P-NMR study

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Using ^{31}P-NMR, we have monitored the metabolic changes in the phosphorus compounds of the appendix, the upper thermogenic part of the inflorescence, of *Sauromatum guttatum* (voodoo lily). Qualitatively, in vivo spectra from D − 6 (6 days before heat-production; the day of heat-production is designated D-day), D − 3, D − 2, D − 1, D-day, D + 1 and D + 2, revealed fluctuations in the concentration of the vacuolar and cytoplasmic inorganic phosphate (P_i) relative to an internal standard. From D − 6 to D − 2 the vacuolar P_i peak was not prominent relative to the other peaks. In the morning of D − 1, an increase occurred in the vacuolar and the cytoplasmic P_i levels. Later on, during development, these levels gradually declined. On D-day, changes in vacuolar and cytoplasmic pH were detected during heat-production. The cytoplasmic pH decreased from 7.7 to 7.2 while that of the vacuole increased by almost one pH unit from 5.8 to 6.7. Perchlorate extract spectra from D − 4, D − 1 and D-day appendices confirmed the observed changes in P_i intensity. These spectra also revealed qualitative changes in the content of ATP and ADP. Their sum decreased during development, reaching its lowest level on D-day at the peak of heat-production between 11:00 and 14:00 (as determined by high performance liquid chromatography of the perchlorate extracts). This suggests that in the early morning of D-day, when heat-production starts, around 9:00, phosphofructokinase, the key enzyme in glycolysis, is not activated by a change in the intracellular ATP/ADP ratio, but by other effector(s).

Introduction

Cyanide-insensitive respiration accounts for more than 90% of the respiration by the appendix of the *Sauromatum guttatum* inflorescence on the first day of inflorescence-opening (D-day). On that day, about 50% of the dry weight of the appendix is metabolized via the non-phosphorylating alternative pathway [19]. Since the electrons flow from the ubiquinone pool to an alternative oxidase and not to the cytochrome oxidase, the oxidative energy is not conserved as ATP in sites II and III of phosphorylation but is released as heat [17,18,24].

The energy status of thermogenic plants has not been studied. The only report on the ATP/ADP ratio in such plants is that of Hess and Meeuse, on *Sauromatum* appendix [12]. They have reported that the ATP/ADP level was lower than 1.5 on D − 1, D-day, and D + 1, a very low value for plant material [11,21,27]. We decided to re-examine these values by using ^{31}P-NMR spectroscopy and high-performance liquid chromatography instead of determination by enzymatic analyses. These techniques have the advantage of comparing the intracellular level of metabolites with their levels in an extract.

The data obtained showed that the ATP/ADP ratio is indeed very low at the peak of heat-production confirming the results of enzymatic assays carried out by Hess and Meeuse [12]. We also found that, on D−1, in the morning, the level of the vacuolar inorganic phosphate (P_i) increased and that on D-day the cytoplasmic and vacuolar pH changed. Furthermore, our data show that the ATP and ADP pools in the appendix are only slightly sensitive to the unusually high metabolic state on D-day. The change in ATP level around noon of D-day does not explain why the glycolytic rate increases in the morning.

Materials and Methods

Plant material

Sauromatum guttatum inflorescences were grown from corms in a growth chamber under 15-h light/9-h dark periods with a photon flux density of 150 μmol

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m^{-2} s^{-1} at 22°C. The developmental stage of the inflorescence was determined relative to the appearance of thermogenicity.

^{31}P-NMR spectroscopy

^{31}P-NMR spectroscopy was performed on a General Electric 7 Tesla high-resolution spectrometer (GE Fremont, CA) at 121 MHz.

Serial NMR spectra were obtained from 3 to 4 cross-sections of the appendix (each section was 8 cm long and weighed 1.5 to 2 g) which were placed so that the volume within the region of the radio-frequency receiver coils of the NMR probe was identical among samples. In the morning, sections were cut from an intact inflorescence, placed in a standard 20 mm NMR tube (Wilmad Glass, Buena, NJ, USA), and its spectrum was immediately obtained. The tissue was aerated by continuous air flow into the NMR tubes at a rate of 21 ml/min. This flow rate replaced the air in the tube every 4 min, and it provided 20-times more oxygen per min than that consumed by 8 g of appendix at the peak of heat-production. Since only 2% of the surface area of the sections was damaged by cutting, the evaporation of water was negligible and the tissue thus was not submerged in buffer. A capillary tube (about 1 mm in diameter) containing 100 mM 2-aminoethylphosphonic acid (δ = 19 relative to H_3PO_4) was placed among the sections in the center of the tube as both a chemical shift reference and standard for normalization of peak heights. The spectra were acquired by using a 10 μs pulse width, 0.243 s recycle delay, 8 K complex points, and 6500 Hz sweep width for a total of 2400 acquisitions. Summed transients were filtered exponentially with 10 Hz prior to the Fourier transform. For each experiment, the magnetic field homogeneity was optimized by shiming on the proton resonance.

Spectra of the neutralized perchloric acid extracts (1 g tissue/4 ml solution) were acquired with 15 μs pulse width, 0.5 s recycle delay, 16 KHz complex points, 8 K sweep width for a total of 23000 scans. Summed data were filtered exponentially with 10 Hz prior to the Fourier transform. Chemical shift values were expressed relative to 2 mM trimethylphosphate (δ = 13 ppm relative to H_3PO_4) added to the extracts as both a chemical shift reference and standard for normalization of peak areas.

Because of the stability of the magnetic field and due to the broad nature of the resonances, the experiments were run without a deuterium lock.

pH determination

Cytoplasmic and vacuolar pH levels were calculated from the chemical shift difference between cellular P_i and published calibration curves for P_i [27,32].

Perchlorate extraction

5 g of freshly cut appendix were ground with a tissue grinder in a solution containing 0.6 M perchloric acid and 1 mM EDTA at 4°C, with a volume ratio of tissue to extraction solution of 1:4. The extract was centrifuged for 10 min at 10000 × g to obtain a clear solution. The supernatant was collected and neutralized with powdered K_2CO_3 until the pH was 7. The

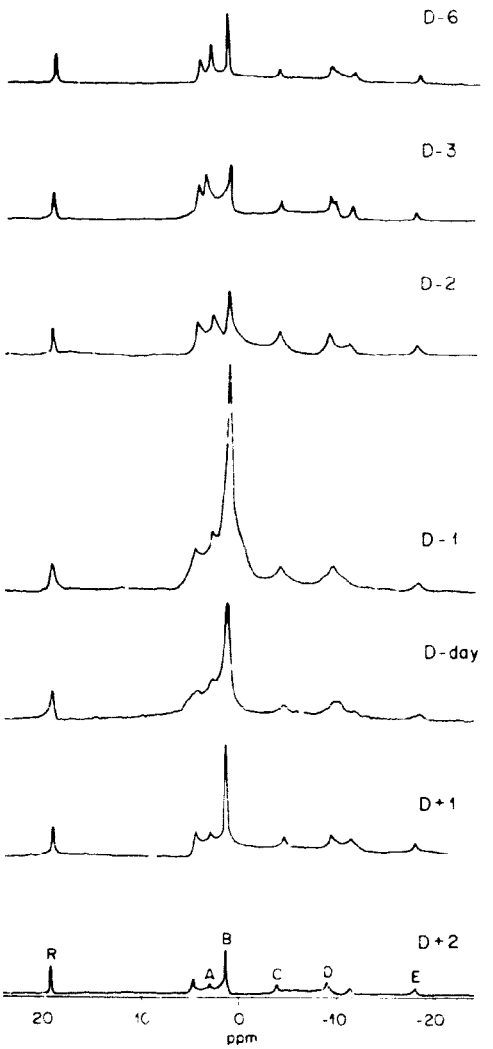

Fig. 1. ^{31}P-NMR spectra of appendices of *S. guttatum* at different stages of development. The stage of development is indicated on the right upper side of each spectrum. The spectra of D−1 and D-day were reproduced with two different appendices. Peak assignments: R, a 100 mM solution of 2-aminoethylphosphonic acid as a reference; A, cytoplasmic P_i; B, vacuolar P_i; C, γ-ATP and βADP; D, α-ATP and α-ADP; E, β-ATP.

precipitated $KClO_4$ was removed by centrifugation and the supernatant was treated twice with Chelex 100 (Bio-Rad, Richmond, CA), at a ratio of 1 g Chelex/4 ml of extract, to remove paramagnetic and other cations. The clear extracts were kept frozen at −10 °C.

Determination of ATP and ADP by HPLC

Fractions taken from the neutralized perchloric acid extracts were analyzed by HPLC using a strong anion-exchange column (VYDAC, Hesperia, CA) and a phosphate gradient from 25 mM (pH 4.5) to 0.5 M (pH 2.7). Peaks were quantified by optical absorbance in comparison with known standards at 210 nm [16].

Determination of P_i by color reaction

Fractions taken from perchloric acid extracts were used for the determination of P_i by a photometric method based on the formation of a complex between phosphomolybdate and malachitegreen [20].

Determination of phosphorus

Dried appendix tissues were digested overnight with concentrated nitric acid. The amount of phosphorus in the digests and standard was determined on an inductively coupled argon plasma-atomic emission spectroscope (ICAP-AES, Jarrel-Ash; 5).

Results

^{31}P-NMR spectra of appendix tissue

The line-widths obtained from in vivo NMR spectra (Figs. 1 and 2) were much broader than those obtained for extracts (Fig. 3); since typical line-widths for this spectrometer are less than 0.01 ppm (proton), we have

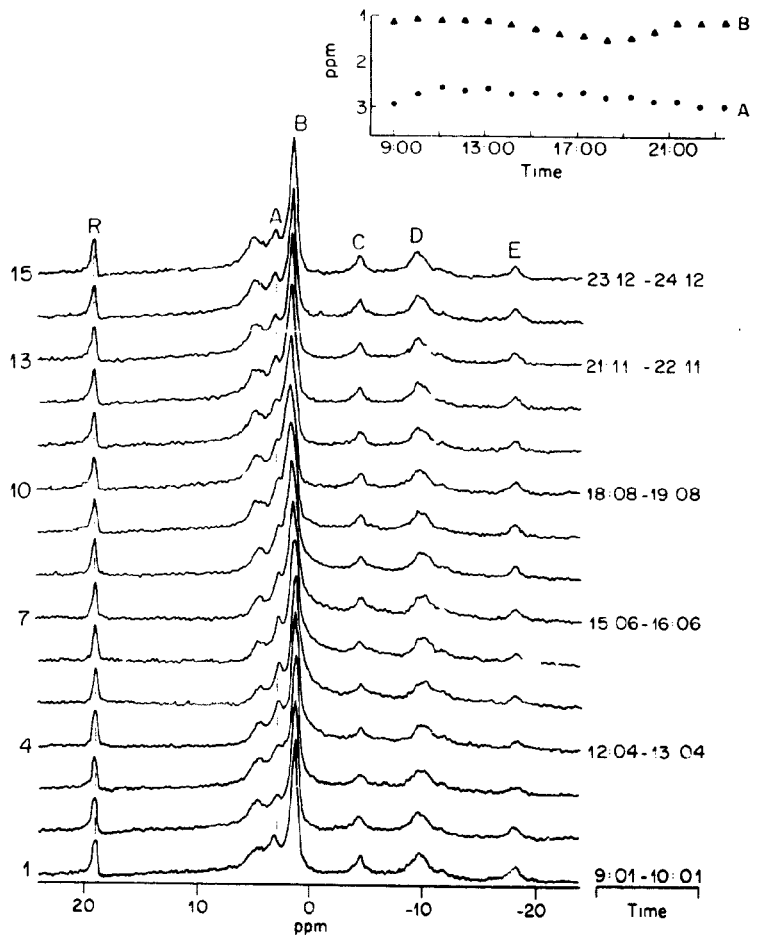

Fig. 2. ^{31}P-NMR spectra of D-day appendix. Fifteen spectra of one out of two appendices are shown. The time of the day is indicated on the right side of each spectrum. The vertical line indicates the chemical shift observed for peak A relative to peak R. Peak assignments as in Fig. 1. The inset in the right corner shows the chemical shifts of the cytoplasmic P_i (peak A) and the vacuolar P_i (peak B) during D-day.

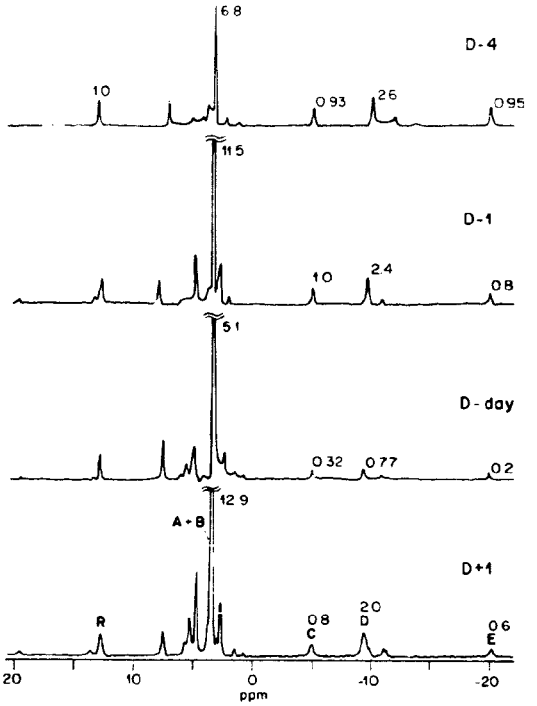

Fig. 3. ^{31}P-NMR spectra of perchlorate extracts obtained from appendices of *S. guttatum* at different stages of development. Peak assignments as in Fig. 1. The number on the right side of the peak indicates the area relative to peak R (2 mM trimethyl phosphate). Each spectrum was obtained from one freshly cut appendix.

concluded that the broad peaks of the in vivo spectra are either a result of heterogeneity in the magnetic susceptibility of the plant material or residual paramagnetic effects. Although infiltration with buffer might have reduced the line broadening due to intracellular air spaces, we decided not to this so because of its adverse effect on heat-production. Consequently, the concentrations of ATP and ADP in vivo could not be determined, and only a qualitative comparison among spectra was made.

A sequence of spectra of appendix tissue before, during and after heat-production is illustrated in Fig. 1. Based on published spectra of plant phosphorus-metabolites [15,27,29], peaks A and B were identified as the cytoplasmic and the vacuolar P_i, respectively, and peaks C, D and E represent ATP and ADP. From D − 6 to D − 2 little difference was found in the peak intensities. In the D − 1 spectrum, the intensity of peaks A and B increased 1.3- and 3.5-fold, respectively, relative to that of D − 2. This increase was not accompanied by a decrease of the same magnitude in the intensity of other peaks. Although these spectra are partially saturated, the increase in the intensity of the P_i peak observed on D − 1 cannot be attributed only to a decrease of the T_1 (spin-lattice relaxation time), since all spectra were acquired under the same conditions. No characteristic 1:2:2:1 splitting pattern of phytic acid at the chemical shift 0 to −2 [15] could be detected, suggesting that the source of the inorganic phosphate was not phytic acid as it is in some other plants [3].

In the D-day spectrum, at the peak of heat production, the levels of all metabolites declined by 50% relative to the D − 1 spectrum. In the D + 1 spectrum, the levels of cytoplasmic and vacuolar P_i stayed unchanged, while that of peaks C, D and E slightly increased relative to the D-day spectrum. In the D + 2 spectrum, the levels of P_i, ATP and ADP were low.

The status of the high-energy metabolites was thoroughly examined during D-day from 9:00 in the morning to midnight (Fig. 2). An assurance that the spectra of such a long experimental period reflect the metabolic status of the tissue was provided when the phosphorus metabolites of a D − 2 appendix were monitored for 16 h by ^{31}P-NMR and the spectra obtained every hour were found to be identical. On D-day, the levels of ATP and ADP dropped considerably between 11:00 and 14:00 (spectra 3–5), following the peak of heat-production in intact plant around 11:00. Later, during the day, the tissue reestablished the ATP/ADP ratio observed at 9:00 in the morning. The fact that the ATP level drops only for a couple of hours suggests that the changes are not due to aging or lack of nutrients but rather are real and reversible. Furthermore, the energy status of the sections is suitable for triggering heat-production; when the sections are treated with salicylic acid and left for almost 24 h on a moist filter with no nutrients, heat develops [25]. Therefore, we can conclude that the drop in the ATP level implies that the cytochrome pathway is unable to respond effectively to an increase in the energy demand that occurs at the peak of heat-production.

The cytoplasmic and vacuolar pH changed during heat-production. The shift in ppm of peaks A and B is shown in the upper right corner of Fig. 2. In the morning, the cytoplasmic pH is 7.74 and the vacuolar pH is 5.68. This is in agreement with other published values of the cytoplasmic and vacuolar pH [32]. Between 9:00 and 12:00, the cytoplasmic pH decreased by 0.5 unit of pH to 7.2. At the same time very little change was observed in the vacuolar pH (around 5.6–5.7). Later during the day, the cytoplasmic pH stayed at around pH 7.3, until late at night, around 23:00, it returned to pH 7.7–7.8. The vacuolar pH fluctuated during D-day. The pH increased from pH 5.68 to pH 6.0 around 13:00, and then it slowly rose to pH 6.7 until 23:00. The changes in pH environments of the P_i-pools are an indication of other processes taking place in the cytoplasm and the vacuole.

TABLE I

Comparison of ATP and ADP concentrations determined by HPLC and ^{31}P-NMR in the appendix of S. guttatum during development

The extracts used for obtaining the NMR spectra shown in Fig. 3 were analyzed by HPLC and the ATP and ADP levels and the ATP/ADP_d ratio were determined. Each value represents one determination. The ATP/ADP_b ratio was determined from Fig. 3. The difference in the area between peak C (β-ADP, γ-ATP) and peak E (β-ATP) is the area of the peak of β-ADP.

	Concentration (nmol/g fresh wt.)			
	D − 4	D − 1	D-day	D + 1
ATP	392	330	138	260
ADP	120	134	148	92
ATP/ADP_d	3.26	2.46	0.93	2.82
ATP/ADP_b	high	4.0	1.66	3.0

TABLE II

The concentration of phosphorus and inorganic phosphate (P_i) in the appendix of S. guttatum during development

The concentration of P_i in the tissue was determined by a color reaction and the concentration of phosphorus by atomic emission spectroscopy. Each value is a mean of two determinations performed on extracts obtained from one appendix. n.d., not determined.

	Amounts (μmol/g fresh wt.)			
	D − 4	D − 1	D-day	D + 1
P_i	1.6	3.2	2.3	3.4
Phosphorus	n.d.	31	32	36

ATP and ADP levels

To confirm that the drop in the intensity of peak C and E is due to changes in ATP and ADP levels, perchlorate extracts were qualitatively examined by ^{31}P-NMR (Fig. 3) and quantitatively examined by HPLC (Table I). From D − 4 to D − 1 small changes in the ATP and ADP levels were observed in both NMR spectra and HPLC data. The resonance of peak E in the NMR spectrum of D − 4 may be a contribution of other compounds; this may be the reason for the very high estimation of the ATP/ADP ratio. The other spectra are in agreement with the HPLC data. On D-day, at the peak of heat-production, the level of ATP dropped by 40% from 330 to 138 μmol/mg fresh wt. while the ADP-level stayed unchanged, around 140 μmol/mg fresh wt., and the ATP/ADP ratio was 1.7 (NMR) and 0.9 (HPLC). On D + 1, after heat-production ceased, the ratio ATP/ADP was around 3.0, when calculated by the two methods. No other nucleotide tri- or diphosphates were detectable in the NMR spectra. The AMP level in the four extracts was low relative to the ATP and ADP levels and was not quantified by HPLC (data not shown). The decrease in the level of ATP on D-day is not followed by an increase in the ADP and AMP levels, suggesting that the adenine nucleotides are degraded. The degradation is irreversible and the D − 1 pool is smaller than that of D − 4, 350 relative to 510 nmol/g fresh wt

The levels of ATP and ADP determined by HPLC are somewhat lower than those found by the enzymatic reactions [12]. However, the results obtained by using the two methods show that on D-day the ATP/ADP ratio is low, close to 1, and the sum of ATP and ADP decreases on D-day and slightly increases on D + 1.

P_i and phosphorus levels

The P_i level determined by a color reaction markedly varied during development (Table II). The amount of total P_i increased two-fold on D − 1 relative to D − 4. It decreased on D-day, and on D + 1 increased again. In the spectra of the perchloric acid extracts (Fig. 3), peaks A and B become one, suggesting that these peaks are the cytoplasmic and vacuolar P_i-pools. The changes in the peak areas of P_i in the extract NMR spectra during development are similar to those determined by the color reaction.

The amount of phosphorus in the appendix tissue stayed relatively constant prior to and during heat-production, suggesting that the changes in the P_i levels are not a result of translocation from other parts of the inflorescence.

Discussion

Although respiration in the thermogenic aroid spadix has been thoroughly studied, very little is known about the energy status of this organ. Hess and Meeuse [12] have shown that at least in 8 appendices the ADP/ATP level was between 1.6 to 0.5 on D-day. Our study shows that in the appendix the ATP/ADP ratio is about 0.9. This ratio is low relative to the 5:10 ratio found in most plants [9–11]. The decrease in the ratio is probably a result of utilization of ATP by energy-consuming processes, and a decrease in the rate of its synthesis. Until D-day, the activity of the cytochrome pathway is much higher than the maximum activity of the cyanide-insensitive pathway [31]. On D-day, the activity of the cyanide-insensitive pathway exceeds that of the cytochrome one [31], and it is followed by a 50% drop in ATP level in the middle of the day, while the ADP level stays unchanged. On D + 1, the activity of the cytochrome pathway is restored and the ATP level increases, while the ADP level decreases. These changes suggest that the operation of glycolysis is not under ATP control in the morning of D-day, because when the ATP-level is unchanged, the glycolytic rate increases. The absence of any relationship between the ATP concentration in the tissue and the operation of the cytochrome pathway is apparent from our data.

The same result has been obtained in maize roots, in which the rate of ATP synthesis was influenced more strongly by the mitochondrial membrane potential than by the concentration of ATP in the tissue [28]. Dry and Wiskich have shown that the control by ATP/ADP of the oxidative phosphorylation ratio is effective only when that ratio exceeds 20 [8].

In the *Sauromatum* appendix, phosphofructokinase is extremely active on D-day [13,14], and it is probably the only control point in glycolysis, as is the case in *Arum italicum* [1,2,6]. The activity of this enzyme is inhibited by high levels of ATP [4,21,26]. Thus, the gradual decline in ATP level from D − 4 to D − 1 presumably increases the rate of glycolysis before D-day. However, the drop of the ATP level, observed only from 10:00 to 14:00 on D-day, does not explain why heat-production starts at around 8:00 in the morning, suggesting that the glycolytic rate increases when ATP level is constant.

Other findings of this study are the increase in the concentration of vacuolar and cytoplasmic P_i in the morning of D − 1, and the fluctuations in the vacuolar and the cytoplasmic pH. It is conceivable that the increase in the P_i concentration of the cytoplasm will stimulate the breakdown of starch and the activity of glycolysis on D − 1. It is well known that P_i is an activator of ADP-glucose pyrophosphorylase [21], an inhibitor of sucrose phosphate synthase [7,22,23] and a regulator of phosphofructokinase [21]. It is likely that an increase in the rate of glycolysis will precede the increase in the level of salicylic acid in the evening of D − 1 [25] and the beginning of heat-production on D-day. A different situation has been found in bean roots, where a decrease in P_i concentration, from 15–20 to 5 μmol/g fresh wt. preceded the development and the operation of the cyanide-insensitive respiration [30].

In summary, the shift on D-day toward the disposal of carbohydrates by the alternative pathway is not followed by dramatic changes in ATP content. The change in the ATP/ADP ratio is slow relative to the dynamic change in the evolution of heat. This suggests that the cytoplasmic ATP/ADP ratio is not the major effector of the activity of phosphofuctokinase, the key glycolytic enzyme that is very active in *Sauromatum* appendix on D-day [12,14]. The role of the adenine nucleotides in carbohydrate metabolism has also been questioned by Hampp et al. [11]. They have reported that the cytoplasmic ATP/ADP ratio does not decrease in transition from light to dark of leaf protoplasts as is expected. In conclusion, the control of glycolysis is probably more complex and not strongly affected by the ATP/ADP ratio.

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