Unidirectional Steady State Rates of Central Metabolism Enzymes Measured Simultaneously in a Living Plant Tissue*

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The unidirectional steady state reaction rates of several enzymes and metabolic fluxes of distinct processes were measured simultaneously in hypoxic maize root tips using two-dimensional phosphorus NMR exchange spectroscopy. A single spectrum monitors ATP synthesis and hydrolysis as well as the activities of four enzymes involved in key pathways of central metabolism: UDP-glucose pyrophosphorylase, phosphoglucomutase, hexose-phosphate isomerase, and enolase. The corresponding unidirectional reaction rates and net metabolic fluxes were calculated from spectral intensities. This method provides a unique picture, at enzyme resolution, of how metabolism reacts in a concerted fashion to changes in external parameters such as temperature and oxygen concentration. By increasing hypoxia via an increase in temperature, we measured the expected increase in glycolysis through enolase activity while total ATP synthesis settled. At the same time, we observed a net flux through phosphoglucomutase and UDP-glucose pyrophosphorylase toward carbohydrate synthesis. This result is discussed in relation to the current hypothesis on the turnover of cell walls and sucrose. This reaction also produces a net flux of pyrophosphate, which is needed by pyrophosphate:fructose-6-phosphate 1-phosphotransferase to work as a glycolytic enzyme.

Understanding the regulation and control of central metabolism in molecular terms is still a global challenge in cell biology. Central metabolism provides the living cells with readily usable forms of energy and with building blocks for biosynthesis. It also plays a major role in the biochemical transformation of cells in response to external signals, or in programmed differentiation. In multicellular eucaryotes, the physiological necessity of biochemical adaptation, including changes in enzyme machinery, is widely recognized and actively studied, and models are available (1). The underlying mechanisms are known to operate at distinct cellular levels, and are characterized by different time constants (2). Schematically, enzyme concentrations are known to change on long time scales, due to processes ranging from gene expression to protein synthesis and degradation. This is referred to as genomic, or coarse, regulation (3–5). The enzyme activities are modulated on medium and short time scales by covalent modifications and in response to variations of metabolite concentrations. This is called metabolic, or fine, regulation (6). There is a permanent interplay between genomic and metabolic regulations, and, following a perturbation, the induced processes may involve a broad range of time scales, from fast allosteric responses to slow changes in enzyme concentrations. In any given physiological condition, the integration of these control and regulation mechanisms eventually leads to a steady state. Stationary as well as transient states are systemic properties of cellular systems. At this level, the metabolic network is usually described with macroscopic variables and analysis is based on the assumption that control is shared between pathways and distributed among enzymes (7). The enzyme concentrations and activities constitute the parameters of the system, while metabolite concentrations and metabolic fluxes are the variables. The variables take values that are a function of the system parameters on one side, of external parameters on the other side. The latter are independent variables and include environmental factors.

Metabolite concentrations and net metabolic fluxes, either at steady state or as transients, are usually measured in cell extracts. With assumptions and modeling, it may also be possible to determine unidirectional fluxes from extract analysis. The net metabolic fluxes determine the function of the cell factory, whereas the unidirectional reaction rates characterize the production units. Both are outstanding dynamic properties of cell metabolism expressing the flow of energy through the cell. The ideal way to find out how they are really distributed is to observe them directly in vivo.

Non-destructive and non-invasive, nuclear magnetic resonance (NMR) can be used to investigate the physiology and metabolism of living systems, from microorganisms to plants (8), from animals to humans (9), and from organelles to organs (10). The method, based upon the observation and characterization of the magnetizations of nuclear spins in different chemical environments, allows identification of molecules in vivo as well as in crude extracts. Absolute concentrations of the more abundant mobile metabolites (11) and their time course during biochemical transformations (12) can be measured in intact cells. Additionally, NMR is the only available method for measuring unidirectional enzymatic fluxes in situ in living systems at steady state (13, 14). This is due to the fact that, for any chemical or enzymatic reaction involving translocation of a nucleus with a spin, magnetization transfer directly reflects mass transfer (15). The unidirectional rate constant of the chemical exchange process can then be measured with NMR if the rate constant is comparable to the longitudinal relaxation rate constants of the exchanging spins. This rather sophisti-
cated method yields invaluable information, since the measured parameters characterize enzyme activities in situ.

Among the NMR techniques used to study chemical exchange, two-dimensional NMR exchange spectroscopy (EXSY)\(^1\) (16) has the potential to monitor several unidirectional enzymatic reactions at steady state in living systems simultaneously. It has only been used to demonstrate the equality of ATP and phosphocreatine fluxes through creatine phosphokinese in rat brain and leg (17) and isolated perfused rat heart (18), but its capacity to measure fluxes of different pathways directly is appealing when studying the regulation of metabolic networks.

The plant cell constitutes a suitable and elaborate model to study the regulation of central metabolism in eucaryotes as a function of external factors using NMR methods (19). Plant cell metabolism has evolved remarkable properties of compartmentation and flexibility in response to terrestrial constraints, and the response and acclimation of higher plants to large variations in environmental factors is spectacular (20, 21). At the molecular level, heterotrophic higher plant cells share a universal strategy to produce ATP and building blocks from oxidation of "fuel" molecules (22–24). Carbohydrates are first converted into a pool of hexose phosphates (25), then transformed through glycolysis into pyruvate (26). Under oxygen deprivation, fermentation is carried out in the cytoplasm (27), whereas, if oxygen is available, the last steps of respiration occur in the mitochondria (28).

The enzymes composing the pathways of central metabolism catalyze numerous chemical reactions where phosphorus atoms change their molecular environment between substrates and products. Using NMR EXSY, we show for the first time that at least 10 of these unidirectional exchange processes can be detected simultaneously in maize root tips. The measurements of the unidirectional fluxes in this plant tissue, as a function of temperature and external oxygen concentration, highlights the acclimation of higher plant cell metabolism to modifications of these environmental factors. ATP turnover and glycolytic flux increase with temperature up to the point where oxygen availability limits the respiratory rate. At 21 °C, in relation to the resumption of growth and increasing hypoxia, we observed an increase in the net flux of glucose 6-phosphate to UDP-glucose synthesis. Since the amount of oligo- or polysaccharides that can be built up from UDP-glucose is far greater than that needed for growth, these observations are discussed in relation to the turnover of sucrose or polysaccharides. The associated production of pyrophosphate (PPi) leads us to suggest that PPi may be used to feed pyrophosphatase: fructose-6-phosphate 1-phosphotransferase acting as a glycolytic enzyme in these hypoxic growing plant cells.

**EXPERIMENTAL PROCEDURES**

**Plant Material**

About 3000 maize (Zea mays L.) seeds (variety DEA, Pioneer France), were soaked for 3 h in aerated running tap water, and arranged on wet filter paper in several layers. During the 3 days of germination, in the dark, at 23 °C, the filter papers were kept humid with a solution of 5 mm Ca (NO\(_3\))\(_2\) in distilled water. Glass beads covering the bottom of the basins prevented the risk of hypoxia from any excess water. Excised root tips, 2–3 mm long, were thoroughly washed in a nutritive medium composed of 100 mm glucose, 5 mm Ca (NO\(_3\))\(_2\), and 1 mm PIPES buffer, at pH 7.0 and 10 °C for 2 h before being used for measurements.

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\(^1\) The abbreviations used are: EXSY, exchange spectroscopy; PIPES, 1,4-piperazinediethanesulfonic acid; Glu-1-P, glucose 1-phosphate; Glu-6-P, glucose 6-phosphate; Fru-6-P, fructose 6-phosphate; UDPG, uridine diphosphoglucose; PEP, phosphoenolpyruvate; PFi, cytoplasmic inorganic phosphate; PGA, phosphoglycerate; PFP, pyrophosphatase: fructose 6-phosphate 1-phosphotransferase; PID, free induction decay.

The sample, typically 6–8 g fresh mass, was then placed in an NMR tube fitted to a tightly closed perfusion system allowing the regulation of pH, temperature, and oxygen content in the circulating medium. A 100 ml/min flow rate was used, renewing the nutritive medium about 14 times/min around the root tips. The respiration rate was monitored continuously during the NMR experiment with a bypass system, allowing measurement of partial oxygen pressure in the medium, successively before and after the sample with the same electrode.

At the end of the NMR measurements, the fresh mass was determined and the sample was frozen in liquid nitrogen for perchloric acid extraction of the metabolites.

**One-dimensional NMR Spectroscopy**

The NMR data were acquired with a Bruker AMX400 WB spectrometer at 162 MHz. D\(_2\)O added to the nutrient medium (0.75% v/v) provided a signal to lock and shim the magnetic field. One-dimensional \(^31\)P spectra of living root tips were registered interleaved with the two-dimensional spectroscopy. They were obtained over 15 min, from the accumulation of 1500 NMR signals (FIDs) with a radiofrequency pulse angle of 45° and a repetition period of 0.6 s. A Lorentz-Gauss apodization was applied to the resulting FID acquired over 2000 points with a frequency window of 5000 Hz.

**Two-dimensional NMR Spectroscopy of Living Root Tips**

**Principle of the EXSY Experiment**—A phosphorus spin is detected first on one molecule, substrate of a given enzymatic reaction, then on a second molecule, product of this reaction. Each spin situation is described by its \(^{31}\)P nuclear magnetization, characterized by frequency and intensity. Between these two observation phases, a flow of magnetization, due to chemical exchange, occurs during a fixed delay called the mixing time. This delay has a typical value of 1.0 s and is the first important time constant of this experiment. It is used to monitor the timing of the enzymatic reaction responsible for the passage of the spin from one molecule to the other. This magnetic labeling is analogous in principle to isotopic or radioactive labeling (12). The NMR technique is not as sensitive as these tracer techniques, but its advantages are outstanding. First, the biochemical system is studied in its natural composition without artificial probes or concentration gradients of labeled molecules. Second, the system is monitored at steady state directly without the need for subsequent assay procedure. Third, the transient non-equilibrium state necessary for observation is introduced on a magnetic property which has no influence upon the chemical functions. Fourth, with a living system at steady state, this transient labeling scheme can be repeated many times to allow detection of a signal. The decay constant of the magnetic label is the relaxation rate constant, R\(_1\), of its magnetization, the order of magnitude for which is 1 s\(^{-1}\).

Due to the small fraction of cytoplasm in a plant cell, the signal-to-noise ratio of an EXSY spectrum is intrinsically low and must be increased by repetition of the elementary acquisition scheme (see below). Thus, the total measurement time necessary to obtain a useful EXSY spectrum, ranging from 12 to 28 h, is the first important time constant of this experiment. The resulting data reflect average values of the unidirectional reaction fluxes detected during this total measurement time, which one-dimensional spectra prove to be at steady state with respect to metabolite concentrations.

**Acquisition Conditions**—The EXSY (or nuclear Overhauser effect spectroscopy) pulse sequence is composed of three 90° radiofrequency pulses and four delays: \(t_2\), 90°, \(t_3\), 90°, \(t_4\), 90°, \(t_1\) (16). Nuclear magnetizations are labeled according to their precession frequency during \(t_1\) and are detected during \(t_2\). The longitudinal components of the magnetization can exchange during the mixing time \(t_m\), and this is the time scale on which the biochemical processes are monitored. For a given \(t_m\) value, the cross-peak intensities in the NMR spectrum are a monotonic function of the rates of magnetization exchange, themselves proportional to chemical fluxes (29). The optimum \(t_m\) value is a compromise between the time necessary for the exchange to occur and the sensitivity loss due to relaxation. In the case of slow exchange, it is approximately equal to the average relaxation time constant \(T_1\) for each cross-peak. In our experiment, the \(T_1\) values of the exchanging resonances measured by inversion recovery ranged from 0.3 s to 3.4 s and an optimum \(t_m\) value was chosen to be 1.0 s.

An elementary two-dimensional spectrum was obtained by repeating the sequence with \(i = 64\) different values of \(t_1\). The relaxation period \(t_2 + t_4\) was set to 1.13 s to maximize signal per unit time. For each value of \(t_1\), \(n = 16\) experiments were added, corresponding to the appropriate phase cycle. Thus, the duration of an elementary EXSY spectrum was 36 min. The absence of \(t_1\) noise in the spectra is a proof of stability of...
both chemical shifts and intensities of the nuclear magnetizations during the elementary, 36-min-long, two-dimensional experiment.

To increase the signal-to-noise ratio, the elementary two-dimensional experiment was repeated $r$ times according to the scheme: $\tau(t_1, t_2, \ldots, t_{2n}, t_{2n+1}, \ldots, t_{2n+1})$. The resulting experimental time ranged from 12 to 24 h, depending on the type of information sought.

Treatment of Data—The raw data were processed with forward linear prediction in the indirect dimension, and Lorentz-Gauss apodization was applied in both dimensions to increase the signal-to-noise ratio without decreasing resolution. Before quantification of the signals, the base plane was corrected. The intensities of cross-peaks and diagonal peaks of each spectrum were measured as integrals over same-sized surfaces (voxels). Similarly, the noise was statistically characterized by the arithmetic mean and standard deviation from 20 different voxels.

Assuming that a steady state of reaction rates was correlated to the observed steady state in metabolite concentrations, we determined these rates from the spectral intensities. Reaction rates were calculated from peak intensities using the procedure of Abel et al. (30), modified for input of the measured relaxation time constants $T_1$. Their measurement can be achieved accurately and precisely when the resonance lines are well resolved in the one-dimensional spectrum and can be achieved accurately and precisely when the resonance lines are well resolved in the one-dimensional spectrum (31). This is not the case of the Glu-1-P line, and the relaxation rate constant of Glu-1-P is difficult to measure in vivo from one-dimensional experiments. We did not take approximate $T_1$ values such as that of the molecule in cell extracts or the $T_1$ value of Glu-6-P in vivo. Instead, for 10 EXSY spectra, we calculated the $T_1$ value, which gives the same net flux value through the enzymes phosphoglucomutase and UDP-glucose pyrophosphorylase. This assumption is valid since Glu-1-P does not accumulate and has no other known major metabolic implications than the ones seen in the EXSY spectra. Furthermore, potential compartmentation effects related to the hexose phosphate pool can be neglected in root tips since plastid development is very limited. The 10 $T_1$ values covered a range of 2.0–3.9 s, giving an average of 2.6 $\pm$ 0.5 s which, by the way, is close to the $T_1$ value of Glu-6-P in vivo. This average value was then used to calculate unidirectional reaction rates of the two quoted enzymes.

Due to the optimized fast acquisition procedure, all the resonances in the spectra (cross-peaks as well as diagonal peaks) were partially and nonuniformly saturated (32). Therefore, all intensities were corrected by a saturation factor taking into account the $T_1$ of the magnetization before exchange, measured in vivo, and the exchange rate constant.

To compare different spectra, each cross-peak intensity was then normalized by the sum of all the intensities in the spectrum, i.e. the total magnetization in the content. This quantity has been estimated as the front surface (voxel). The resulting experimental time ranged from 12 to 29 h, depending on the type of information sought.

RESULTS

Bioenergetic Steady State of the Living Tissue

One-dimensional $^{31}$P NMR spectra, commonly used to characterize the energetic status of living cells (33), give a static overview of cellular energetics. They were used here to monitor the stability of the sample’s energetic status over the whole time needed to acquire the EXSY data. The chemical shifts of the one-dimensional spectrum (Fig. 1A, spectrum 1) were interpreted from published data (34, 35) and, in case of resonance overlap, were confirmed by analysis of the corresponding in vitro spectrum (Fig. 1A, spectrum 2). When necessary, identification was achieved by observing co-resonances on addition of pure compounds to the extract, and the pH titration behavior of the chemical shifts. Heteronuclear $^{31}$P–$^1$H chemical shift correlation spectroscopy (36) was used to ascertain the existence and position of the resonance of glucose 1-phosphate in the one-dimensional $^{31}$P spectrum of living root tips (Fig. 1B). For Glu-1-P, the chemical shift of the anomeric proton magnetically coupled to phosphorus is indeed very characteristic.

The resonance positions and intensities of the one-dimensional NMR $^{31}$P spectra, interleaved between $^{31}$P EXSY spectra, were stable throughout the duration of the two-dimensional experiment, which was 12–29 h in general. The intensities being proportional to the concentrations of phosphorylated metabolites, their constancy was taken as a criterion to ascertain that the tissue was at steady state with respect to energetics during the total experimental time.

Exchange Spectroscopy of Maize Root Tips

Fig. 2A shows a typical phosphorus EXSY spectrum of living maize root tips, where discrete peaks are characterized by two frequencies and one intensity, indicated by contour lines. The frequency on the vertical axis indicates the origin of the magnetization, when the spins belong to the substrate. The horizontal axis gives the frequency of the magnetization after the mixing time, when the spins are part of the product. The peaks on the diagonal have the same substrate and product frequencies. They correspond to magnetizations or fractions of them which have not been exchanged during the mixing time. Their intensities are different from a one-dimensional phosphorus spectrum, but their positions are identical, and the assignment of the diagonal peaks is identical to the resonance assignment of a one-dimensional spectrum. The substrate and the product frequencies differ for the peaks outside the diagonal, called cross-peaks. They are attributed to chemical exchange of phosphorus atoms between different molecules, during the mixing time, through enzyme activity. The cross-peak intensities are interpreted as magnetization flows, linked to mass flow, between the two diagonal species they interconnect. Each cross-peak is assigned to a reaction on the basis of the chemical shift of the two exchanging resonances and on established biochemical data (Fig. 5) (37–39). In this way, we were able to assign the following chemical exchange processes and related enzymatic activities.

Enzymatic Reactions Observed in Situ

ATP Hydrolysis and Synthesis—Since the phosphorus resonances of the different nucleotide triphosphates (NTP) are not resolved in vivo, peak A corresponds to phosphorus spins in ATP, or other NTP hydrolase that release inorganic phosphate into the cytoplasm. Peak B corresponds to the reverse process, the transfer of P$_i$ to γ-NTP. This occurs only during ATP synthesis and, in the presence of oxygen, it essentially reveals the activity of mitochondrial ATP synthase (40).

UDP-glucose Pyrophosphorylase—Peaks C and D correlate the diagonal peak of uridine diphosphoglucose (UDPG) β-phosphate with the group of resonances that includes the resonance of cytoplasmic inorganic phosphate (Pi). These resonances are resolved in acid extract spectra and attributed without any ambiguity to inorganic phosphate (Pi), glucose 1-phosphate (Glu-1-P), and myo-inositol hexakisphosphate (phytate). Therefore, the most direct interpretation of cross-peaks C and D is that they arise from the chemical exchanges of phosphorus atoms between UDPG and Glu-1-P. They identify the reactions catalyzed by uridine triphosphatase:glucose-1-phosphate uridylyltransferase (UDP-glucose pyrophosphorylase, EC 2.7.7.9): Glu-1-P + UTP $\rightarrow$ UDPG + PP$_i$. A two-step exchange from UDPG to Glu-1-P to P$_i$ to explain peak C is ruled out because the activities of cytosolic hexose-phosphate phosphatases in this material cannot account for such an intense cross-peak. Furthermore, precise measurements of the positions of cross-peaks A and C show clearly a difference in their absorptions. This had also been demonstrated previously, using frequency dependent saturation transfer (41).

Enzymes of the Hexose Phosphate Pool—The cross-peaks E and F are attributed to the interconversion between glucose 6-phosphate (Glu-6-P) and Glu-1-P, catalyzed by phosphoglucomutase (EC 5.4.2.2). Cross-peak F cannot be explained by cytosolic glucose 6-phosphate activity for the reasons mentioned above. Peak G arises from the two-step reaction Glu-6-P...
Glu-1-P \rightarrow \text{UDPG, catalyzed by the enzymes responsible for peaks F and D. Note how this two-step exchange is characterized by a much lower intensity than the one-step exchange.}

The cross-peaks L and M (Fig. 2B) correspond to the exchange between Glu-6-P and fructose 6-phosphate (Fru-6-P) catalyzed by phosphohexose isomerase (phosphoglucose isomerase, glucose-6-phosphate isomerase, EC 5.3.1.9). Because of their proximity to the diagonal, these peaks are only clearly resolved in spectra acquired with high frequency resolution and proper quantification is more difficult to achieve.

Glycolytic Enzymes—Phosphoenolpyruvate (PEP) is the only abundant metabolite with a resonance around 21 ppm in 31P NMR spectra of acid extracts. The cross-peak H is therefore attributed to the synthesis of PEP. However, the chemical shift of the substrate molecule corresponds rather to 3-phosphoglycerate (3-PGA) than to the expected 2-phosphoglycerate (2-PGA). We thus observe a two-step exchange process from 3-PGA through 2-PGA to PEP, catalyzed by phosphoglyceromutase (EC 2.7.5.3) and enolase (EC 4.2.1.11). In this case, the 2-PGA intermediate is not directly observed, probably due to its lower abundance and short lifetime. Another explanation might be metabolic channeling of 2-PGA through the two enzymes. The absence of the symmetric counterpart indicates that the rate of PEP synthesis is higher than the rate of PEP consumption by the reverse reaction.

Response of Reaction Rates to External Parameters

Since the magnetization of a particular species is proportional to its concentration, magnetization fluxes are propor-
The constancy of the three NTP resonance intensities in the living sample per second. The error bars represent total uncertainties coming mainly from spectral noise and to a lesser extent from $R_1$ uncertainties. $A$, empty circles represent total ATP synthesis rates in the root tips; filled circles represent ATP hydrolysis rates which measure mainly the ATPases activity. The difference between the two sets of rates represents the ATP used elsewhere in the cell, mainly in phosphorylation and biosynthesis processes. $B$, phosphoglyceromutase and enolase rates. Each point measures the rates of interconversion between 3-PGA and PEP since 2-PGA, the intermediate metabolite, is not detected. There is a lack of data (mainly $T_1$) to calculate a normalization factor for the rate scale which is empirically adjusted. The differences between the unidirectional rates represent the net flux through the glycolytic pathway.

**FIG. 2.** 162 MHz $^3$P NMR EXSY spectrum of living maize root tips. Sample at 20 °C, perifused with a 100 mM glucose medium at pH 7.0, saturated with air. $A$, $t_m = 1.0$ s and $t_d + t_f = 1.13$ s. An elementary experiment is obtained by accumulating 16 FIDs for each of the $i = 64$ $t_i$ points over a period of 36 min. This is repeated 20 times, and the sum of the elementary two-dimensional spectra constitutes the final two-dimensional spectrum acquired in 29 h and 16 min. $B$, $t_m = 3.0$ s and $t_d + t_f = 3.03$ s. The number of $t_i$ values is $i = 192$, and $n = 4$, yielding a final high resolution spectrum in 20 h and 41 min.

**FIG. 3.** Variations in enzymatic reaction rates as a function of temperature for maize root tips perifused with a 100 mM glucose medium saturated with a gas mixture of 5% $O_2 + 95% N_2$ at pH 7.0. The rates are expressed in fractions of the total mobile phosphate content of the living sample per second. The error bars represent total uncertainties coming mainly from spectral noise and to a lesser extent from $R_1$ uncertainties. $A$, empty circles represent total ATP synthesis rates in the root tips; filled circles represent ATP hydrolysis rates which measure mainly the ATPases activity. The difference between the two sets of rates represents the ATP used elsewhere in the cell, mainly in phosphorylation and biosynthesis processes. $B$, phosphoglyceromutase and enolase rates. Each point measures the rates of interconversion between 3-PGA and PEP since 2-PGA, the intermediate metabolite, is not detected. There is a lack of data (mainly $T_1$) to calculate a normalization factor for the rate scale which is empirically adjusted. The differences between the unidirectional rates represent the net flux through the glycolytic pathway.

**Steady State Rates of Enzymes in Vivo**

**ATP Synthesis and Hydrolysis**—In a living cell at steady state, synthesis and consumption of NTP are balanced precisely. The constancy of the three NTP resonance intensities in one-dimensional spectra recorded throughout the EXSY experiments (data not shown) indicates that the root tips remained at steady state during the whole duration of the experiment. The upper curve of Fig. 3A shows the total flux of ATP synthesis from ADP and P$_i^C$. The lower solid curve gives the flux of NTP hydrolysis to NDP and P$_i^C$. Thus, the difference between the two curves (dotted curve) measures all NTP-consuming processes that do not lead to NMR-visible P$_i$, i.e. like phosphorylations and biosynthetic reactions yielding pyrophosphate. ATP synthesis increases as a result of thermal activation at low temperatures, but rapidly levels off at higher temperatures because mitochondrial oxidative phosphorylation is limited by the amount of available oxygen ($pO_2 = 5\%$).

**Glycolysis**—Fig. 3B displays the exchange processes between 3-PGA and PEP, through 2-PGA, which is not detected. Each exchange rate, arising from two enzymatic reactions, increases with temperature as a result of thermal activation. Their absolute values cannot be calculated because the relaxation rate constants $R_i$ of the exchanging spins are unknown. The net glycolytic flux also increases as a function of temperature, partly as a consequence of increasing hypoxia.

**UDP-glucose Metabolism**—The rates catalyzed by phosphoglucomutase and UDP-glucose pyrophosphorylase behave differently as a function of temperature (Fig. 4, A and B). The forward rates of the two enzymes (open circles) considerably double between 16 and 21 °C but do not change much in the low temperature range between 11 and 16 °C. The reverse rate of UDP-glucose pyrophosphorylase does not appear to be sensitive to temperature, while the reverse rate of phosphoglu-
comutase increases steadily with this factor (closed circles). At 21 °C, the absolute value of the unidirectional rates catalyzed by the two enzymes are significantly different, the rates of interconversion of hexose phosphates being 50–100% higher than the rates of UDP-glucose pyrophosphorylase. There was no evident net flux at the low temperatures, but clearly a net interconversion of hexose phosphates being 50–100% higher than the rates of UDP-glucose pyrophosphorylase. All given error bars contain the cumulative effect from all potential sources of uncertainty using Gaussian error propagation. Overall, the sensitivity and precision of our experiments are such that we can observe changes in the detected reaction rates in response to temperature variations as partial oxygen pressure was fixed in the nutritive medium (Figs. 3 and 4). Although the first two sets of data (Fig. 3) validate the method on known effects, the third set (Fig. 4) yields new information on higher plant metabolism in hypoxia.

EXSY spectra can yield the exact resonance frequencies of peaks that are overlapping in the corresponding one-dimensional spectra (especially Glu-1-P but also 3-PGA and Fru-6-P). The one-dimensional spectral resolution deduced from the two-dimensional spectra of a living sample is comparable to the one-dimensional spectral resolution obtained with the tissue acid extract. In fact, if they were unambiguously identified on a biochemical basis, the EXSY cross-peaks could be used to determine with precision the chemical shifts of phosphorylated metabolites in one-dimensional spectra. Increasing the two-dimensional spectral resolution further during acquisition of the data is time consuming.

Sensitivity is the most limiting factor for routine use of the EXSY technique since, at a given magnetic field, the signal-to-noise ratio is proportional to the square root of the experimental time. Using 6–8 g of root tips and a 9.4 tesla spectrometer, 12 h of experimental time was sufficient to obtain a usable EXSY spectrum, and all the spectra quantified for the present report were acquired in 29 h. Assuming that a steady state of metabolic fluxes was correlated to the observed steady state in metabolite concentrations, we determined the unidirectional reaction rates of the biochemical exchange processes from the spectral intensities measured as volumes of the cross-peaks. The EXSY signal is also proportional to the total enzymatic rate and thus to the amount of cells. Under the present experimental conditions the lower limit for a reaction rate to produce an NMR EXSY signal is estimated to be less than 10 nmol/s/g of fresh mass.

The main source of error in the calculated rates and fluxes stems from the uncertainty in the cross-peak intensities due to spectral noise. The generous allowances for uncertainties in the relaxation rate constants $R_1$ have a notable effect on the uncertainty of only some rates, namely the ATP synthesis rate, the rate from Glu-6-P to Glu-1-P and on the net flux of UDP-glucose pyrophosphorylase. All given error bars contain the cumulative effect from all potential sources of uncertainty using Gaussian error propagation. Overall, the sensitivity and precision of our experiments are such that we can observe changes in the detected reaction rates in response to temperature variations as partial oxygen pressure was fixed in the nutritive medium (Figs. 3 and 4). Although the first two sets of data (Fig. 3) validate the method on known effects, the third set (Fig. 4) yields new information on higher plant metabolism in hypoxia.

The spectral intensities observed are the result of the magnetization fluxes accumulated over the total duration of an experiment. Thus, we did not measure instantaneous unidirectional reaction rates of individual enzymes, but the integral of these rates, over approximately 1 day. Since the concentrations of the reactants remained constant over this time, any significant change in rates during their measurement is unlikely. On the contrary, we observed that a new steady state was reached rapidly (approximately within 20 min) when the temperature of the sample was changed. Thus, our rate measurements characterize asymptotically stable steady states (43), resulting from coarse and fine regulation processes operating in vivo. They characterize a biochemical adaptation very distinct from the immediate response following the variation of the environmental factor. The internal structure could be reorganized during this long period of time, resulting in conservation of function, pictured as metabolic fluxes.

**Enzymes of Sucrose Metabolism**—In higher plants, the respiratory substrate of non photosynthetic cells is sucrose, transported through the phloem from source to sink organs. Sucrose metabolism in non-photosynthetic plant cells is depicted in Fig. 5. Sucrose can be cleaved to glucose and fructose by an invertase. Using NMR EXSY, we have detected, assigned, and quantified 10 unidirectional exchange processes of phosphorus atoms involved in the reactions of energetic metabolism in a living plant tissue. The reliability of our measurements and their relevance to metabolism need to be assessed before discussing the biological implications of our findings.

**DISCUSSION**

**Resolution, Sensitivity, and Relevance of EXSY Experiments**—Using NMR EXSY, we have detected, assigned, and quantified 10 unidirectional exchange processes of phosphorus atoms involved in the reactions of energetic metabolism in a living plant tissue. The reliability of our measurements and their relevance to metabolism need to be assessed before discussing the biological implications of our findings.
Steady State Rates of Enzymes in Vivo

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S. Phosphofructokinase; A. Aldolase; E. Phosphoglyceromutase; C. Phosphoglucomutase; G. UDP-glucose pyrophosphorylase.

Reactions would depend upon the concentration of the different substrates including PPi. In normoxic maize root tips, it was concluded from NMR saturation transfer measurements that UDP-glucose pyrophosphorylase was catalyzing a near-equilibrium reaction (41).

Our results show that in hypoxic glucose-fed maize root tips the ratio between the forward and reverse rates of UDP-glucose pyrophosphorylase is close to 1 at the low temperatures and is around 2 at 21 °C (Fig. 4B) with a net flux, \( v_f/v_r \), toward UDPG synthesis (Fig. 4C). In chemical thermodynamics the disequilibrium ratio of a reaction \( \rho = 1/K_{eq} \) (unit mass-action ratio and \( K_{eq} \), equilibrium constant) gives a measure of the displacement of the reaction from equilibrium (2). Kinetics equations show that \( \rho = v_f/v_r = 1 - v_f/v_r \), where \( v_f \) and \( v_r \) are, respectively, the forward and reverse rates of the reaction, and \( v_f \), the net flux through the reaction. Our results yield \( \rho \approx 2/3 \) for phosphoglucomutase and \( \rho \approx 1/2 \) for UDP-glucose pyrophosphorylase in the maize root tips at 21 °C. This indicates that, even at 21 °C, the UDP-glucose pyrophosphorylase reaction is close to equilibrium, which is consistent with earlier studies indicating that the activity of this enzyme is much higher than that of other enzymes involved in polysaccharide biosynthesis (25).

In the present case, the function of UDP-glucose pyrophosphorylase is clearly not to degrade UDPG formed by sucrose synthase, as usually assumed for sucrose utilizing tissues, but rather, at least at 21 °C, to synthesize UDPG from hexose phosphates. Then the question arises whether glucose feeding induces an artificial metabolism in the maize root tips, with no need for sucrose synthase to catabolize sucrose. We believe this is not the case, and that the observed metabolism of sucrose is likely to reflect the real situation for the following reasons. (i) The carbon supply of maize root tips in plants is not only sucrose but also hexoses produced by the hydrolysis of sucrose in the apoplast (46). (ii) Sucrose has been shown to be metabolized by sucrose synthase in tissues which receive only hexoses, instead of sucrose, as carbon substrate (47). (iii) In tissues supplied with either glucose or fructose, labeling studies have shown that sucrose synthase is active in both directions (48). Therefore, since glucose-fed excised root tips contain sucrose, it is likely that their sucrose metabolism reflects the metabolism of root tips in plants.

Pyrophosphate Metabolism—PPi plays a major role in the energy metabolism of higher plant cells (25–27, 49). As in other organisms, it is a by-product of biosyntheses. However, in plants, acid and alkaline pyrophosphatases being located in the vacuole and plastids exclusively, cytosolic PPi is not hydrolyzed. Concentrations are in the range 0.04–0.8 mM, and PPi can be used as an energy donor, instead of ATP, in a variety of situations (50). It may be a substrate of the vacuolar PPi-dependent proton pumps and of the cytosolic enzyme pyrophosphate/ fructose 6-phosphate 1-phosphotransferase (EC 2.7.1.90) (PFP), also called PPi-dependent phosphofructokinase. This enzyme catalyzes the reversible conversion of Fru-6-P to fructose 1,6-bisphosphate in parallel with the reaction catalyzed by 6-phosphofructo-1-kinase (EC 2.7.1.11). Of importance here, PPi can also be consumed by the UDP-glucose pyrophosphorylase reaction, when sucrose is degraded, or produced by this enzyme during sucrose synthesis. Both the PFP and UDP-glucose pyrophosphorylase reactions are readily reversible in vivo, and the cytosolic PPi concentration may determine the direction of these reactions (Fig. 5) (25, 51).

In hypoxic maize root tips at 21 °C, there is a net flux from Glu-6-P to Glu-1-P to UDPG (Fig. 4C), which appears to be of the same order of magnitude as the flux needed for PFP to operate in the glycolytic direction (Fig. 3B). In this situation, it is likely that UDP-glucose pyrophosphorylase would maintain...
the sustained production of PPI used to activate the glycolytic flux through PPF. The relative limitation in ATP turnover (Fig. 3A) and lowering of ATP content (Table I) that occurs in the hypoxic plant tissue would also explain that PPF is contributing to the glycolytic flux more efficiently than phosphofructokinase. This interpretation is supported by the fact that the specific activity of PPF in glucose-fed excised maize root tips increases by about 25% after 6 h of anoxia (52). It also increases considerably in suspension cultures and whole plantlets of rice grown under anoxia, in contrast with a concomitant stabilization of phosphofructokinase activity (53, 54). It is also coherent with the kinetic properties of the enzyme, at least as far as the maize root enzyme resembles that of potato tuber (55). A possible excess of PPI could be used by the PPI-dependent proton pumps of the tonoplast (27), thus contributing to the avoidance of the cytoplasmic acidosis induced by oxygen deprivation.

Cycling of Carbohydrates—The net flux of UDPG observed at 21 °C must be used for carbohydrate synthesis. Fig. 4C shows that this flux is 2.6–10 mol per second, which is the second total mobile phosphorus content of the sample of 38 μmol, as measured in an acid extract at the end of the actual experiment. Then, the net flux of UDPG formation is around 99 mmol s−1. For a 29-h experiment at 21 °C, this would amount to about 2 g of glucose. The fresh mass increased from 6.3 g to 10.4 g at 21 °C. This 4 g difference corresponds roughly to 0.4 g of dry matter, i.e. synthesis of polysaccharides. This estimation leaves us with about 1.6 g of glucose-equivalent as a consequence of the net forward flux catalyzed by UDP-glucose pyrophosphorylase, which is not accounted for in biomass production. Some storage of soluble sugars was observed in roots of maize, both in hypoxic (56) and normoxic conditions (57), but this is too small to account for the net flux to UDPG. Furthermore, one-dimensional 13C NMR measurements show a slight increase, if any, in sucrose concentration (data not shown). Our conclusion, then, is that the carbohydrate flux detected is mainly due to substrate cycling. Some cell wall turnover cannot be excluded (58, 59), but this result is in keeping with other data on sucrose cycling. Sucrose would be formed from UDPG and Fru-6-P through the reaction catalyzed by sucrose-phosphate synthase, and split into glucose and fructose with a cytosolic invertase (Fig. 5) (60, 61). The quantitative importance of this futile cycling appears to vary according to tissues. In soybean cotyledons this was only around 3% (62), whereas in maturing banana (63) and in normoxic root tips (57) it was found to consume most of the ATP produced by respiration. Our EXSY experiment directly measures the activity of enzymes involved in de novo synthesis of carbohydrates, and allows a direct comparison with the flux of ATP synthesis or degradation. Since these fluxes appear to be of the same order of magnitude (Table II), our results confirm previous data suggesting that sucrose (and cell wall) cycling make a significant contribution to ATP consumption in heterotrophic tissues even in energetically unfavorable hypoxic conditions. It has been proposed that this apparent futile cycle of sucrose synthesis and degradation would allow the cell metabolism to shift easily from a function of sucrose production to a function of sucrose degradation, after receiving elicitor signals. We would also like to mention that it could be an important new regulatory mechanism for the osmotic properties of the cell.

**Contribution of EXSY to Understanding Metabolic Control**—These results confirm the potential of NMR chemical exchange techniques to measure directly in their cellular environment the unidirectional reaction rates of enzymes. This information is very different from that obtained when measuring enzyme activities in extracts since common biochemical assays determine the maximum possible activity of enzymes. Furthermore, the forward and backward rates catalyzed in situ by an enzyme can be obtained from a single EXSY spectrum, and, from their knowledge, it can be determined how far the reaction is from equilibrium. Additionally, the difference between these two rates readily yields the net flux through the enzyme pathway, a systemic property of the metabolic network, as opposed to the molecular property of its individual components.

By revealing multiple chemical exchanges catalyzed by different enzymes, NMR EXSY displays a global picture of ongoing metabolism. With the simultaneous observation of enzyme activities from different metabolic pathways, the experiment gives access to their coordinated function in vivo. From the results discussed above, one can imagine how EXSY may contribute further to give a picture of the metabolic acclimation of higher plant cells to modifications of dominant environmental factors. It does so by combining in a single experiment the two main strands of metabolic biochemistry: the system level and the molecular level.

The substrate cycling phenomenon may be one of the dynamic regulatory mechanisms underlying energetic homeostasy in higher plant cells. In fact, the sucrose cycle, along with several others, had been foreseen years ago as a potential futile cycle in the carbohydrate pathway of central metabolism of eucaryotic cells in the resting state (64). Because they are noninvasive and sensitive to flux, magnetization transfer techniques appear particularly well suited to investigate this kind of dynamic process in living systems. The experiments can be interpreted more readily than isotope labeling experiments whose data are more difficult to unravel in such systems.

Direct measurement of key reaction rates in vivo by magnetization transfer could also provide a way to tackle questions linked to the reorganization of central metabolism following large internal perturbations. For instance, biotechnology has now documented several cases where induced transgenic mutations leading to the desired overexpression of enzyme did not
give the expected output fluxes or phenotype because of the unexpected adaptation of central metabolism to this modification (65–67). By analogy to what is shown here, it can be inferred that NMR EXSY also offers the opportunity to discover the redistribution of main fluxes after substantial changes of enzyme concentrations and/or activity, whether they originate from repression or overexpression.

The main stages of cellular respiration: sucrose metabolism, glycolysis, and ATP turnover, can be studied simultaneously in a meristematic tissue using 31P NMR chemical exchange spectroscopy. With continuous improvements in NMR sensitivity, the development of proton, carbon, and nitrogen spectroscopy to detect other metabolites should provide probes for other metabolic pathways in plant cells. When feasible, NMR EXSY could yield unique information about how the concerted reaction of metabolism is organized in response to environmental fluctuations and stresses on the one hand, to xenobiotics and pathogens on the other.

This experimental method has also broad ranging implications as a tool for studying metabolic regulation in a variety of other living systems. It opens a new window on the molecular behavior of integrated systems by displaying the activities of an ensemble of enzymes acting together in their natural environment. It offers an opportunity to study the control processes that are active in a given steady state by actually measuring a whole set of unidirectional enzymatic rates. However, these rates must be large enough to create detectable flows of magnetizations following chemical exchange. Flux measurements will be facilitated if the intrinsic signal-to-noise ratio of the one-dimensional NMR spectra is high. This can be the case with microorganisms and animal cells, tissues, and organs where cytoplasmic volume or cell density are often larger than in plant materials.

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