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PROBING PLANT METABOLISM WITH NMR

R George Ratcliffe
Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, United Kingdom; e-mail: george.ratcliffe@plants.ox.ac.uk

Yair Shachar-Hill
Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, New Mexico 88003; e-mail: yairhill@nmsu.edu

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■ **Abstract** Analytical methods for probing plant metabolism are taking on new significance in the era of functional genomics and metabolic engineering. Among the available methods, nuclear magnetic resonance (NMR) spectroscopy is a technique that can provide insights into the integration and regulation of plant metabolism through a combination of in vivo and in vitro measurements. Thus NMR can be used to identify, quantify, and localize metabolites, to define the intracellular environment, and to explore pathways and their operation. We review these applications and their significance from a metabolic perspective. Topics of current interest include applications of NMR to metabolic flux analysis, metabolite profiling, and metabolite imaging. These and other areas are discussed in relation to NMR investigations of intermediary carbon and nitrogen metabolism. We conclude that metabolic NMR has a continuing role to play in the development of a quantitative understanding of plant metabolism and in the characterization of metabolic phenotypes.

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INTRODUCTION

The current renaissance in the investigation of plant metabolism is being driven by two factors. First, the continuing advances in molecular genetics are creating unprecedented opportunities for dissecting the operation of metabolic pathways. For example, the increasingly facile transformation of plants is providing a powerful tool for exploring the contribution of specific enzymes to the control of metabolic flux, as well as generating increasing demand for the characterization of metabolic phenotypes. Second, progress in the development of new tools for the quantitative analysis and modeling of pathways is permitting increasingly sophisticated investigations of the integration of metabolism. Taken together these two factors have invigorated the field and raised the profile of plant metabolism in plant biology (22).

Despite this renaissance, the fundamental metabolic questions that need to be answered remain much the same. These questions revolve around the identity and quantity of the metabolites that are present, the identification of the pathways that link them, and the factors that allow the resulting metabolic fluxes to be controlled. Moreover, the underlying pattern of gene expression is expected to lead to answers that are generally both spatially and time dependent. Thus while the revolution in molecular genetics has provided new tools for addressing these questions, and new contexts in which they need to be answered, the ultimate goals of contemporary metabolic analysis are really little different from the targets first identified by the pioneers of metabolic research when they embarked on the study of fermentation in yeast (28).

Analytical tools are the key to answering metabolic questions. Accordingly, this review describes the contribution of a versatile technique, nuclear magnetic resonance (NMR) spectroscopy, to the investigation of plant metabolism. The evolution of this particular application of NMR can be traced back to the ground-breaking ^{13}C NMR studies of Schaefer & Stejskal on carbohydrate and lipid metabolism (140, 141). The subsequent development of the technique and its applications have been charted at regular intervals in the review literature (81, 111, 117, 120, 144, 146), and although not as widely exploited as its proponents might wish, NMR is now an established technique in the armory of the plant biochemist.

This review focuses on the metabolic applications of NMR in higher plants, with the emphasis on the pathways of primary metabolism. We therefore largely ignore secondary metabolites, despite the fact that NMR has long been used for the elucidation of their synthesis in plants and other organisms (13, 151). We also ignore a number of nonmetabolic areas in which NMR contributes to our

understanding of plant biology. These areas include the investigation of water and water movement in plants, which are frequently studied by low resolution ^{1}H NMR (157) and NMR imaging (26, 85, 111); the many contributions of NMR spectroscopy to macromolecular structure determination; the investigation of plant ultrastructure and root architecture by NMR imaging (26, 85, 111); and the rapidly developing application of solid-state NMR methods to the characterization of plant cell walls (44, 55, 67).

IDENTIFYING AND QUANTIFYING METABOLITES

Primary metabolites are composed of hydrogen, carbon, nitrogen, oxygen, phosphorus, and sulfur. All of these elements have magnetic isotopes detectable by NMR, and with the exception of sulfur, the resulting signals can be used to identify metabolites in plant tissues and their extracts. The signals are observed by placing the sample in the magnet of an NMR spectrometer, and the resulting spectrum often contains sufficient information to identify and quantify the metabolites that are present. Moreover, since the measurement is nondestructive, it is possible to record a series of in vivo spectra from the same sample, and then to interpret the time course in terms of the metabolic response of the plant material to any change in its physiological state.

Full details of the practical aspects of recording and interpreting plant tissue NMR spectra are readily available elsewhere (111, 113, 129, 146). Suffice it to say that interpretable spectra can be recorded from a wide variety of material, including cell suspensions, excised tissues, and intact seedlings, and that it is possible to impose a range of physiological conditions on such samples within the confines of the NMR magnet. The sensitivity of the experiment is extremely variable, since it is strongly dependent on the nature of the sample, the isotope that is detected, the magnetic field strength of the spectrometer, and the way in which the NMR signals are recorded. As a very rough guide, a reasonable spectrum can often be obtained in 30 minutes from the metabolites present at millimolar concentrations in a 1 g sample, but this estimate could easily change by an order of magnitude according to the nature of the experiment. For example, ^{13}C-labeled fermentation products have been detected within a minute in a maize root tip sample weighing only 0.1 g (148). In contrast, it may take 12 h or more to define the unlabeled ^{13}C NMR signals from metabolites present at millimolar concentrations in extracts prepared from several grams of tissue. These considerations indicate that the practicality of an in vivo NMR experiment is often best judged empirically.

The magnetic properties of the detectable isotopes are very different, and this results in significant differences in the utility of the corresponding NMR spectra. Nevertheless, characteristic signals can be detected from many metabolites (34), and in some cases, in vivo NMR analysis has even led to the detection of new or unexpected compounds (35, 52, 134, 135). The salient features of the most useful NMR approaches are summarized in the following paragraphs. Note that the

description is restricted to the specific role of NMR spectroscopy in identifying and quantifying metabolites, and that subsequent sections of the review consider the ways in which the same techniques can be used for other purposes.

Metabolite Detection Strategies

The hydrogen atom has three magnetic isotopes—^{1}H, ^{2}H, and ^{3}H—but only ^{1}H is relevant here. The high sensitivity of ^{1}H NMR, which stems from the favorable magnetic properties of the ^{1}H isotope and its 99.985% natural abundance, is a considerable advantage, but the usefulness of the technique is somewhat reduced by the overlapping of the signals from the detectable metabolites. This problem can be alleviated by the use of two-dimensional NMR experiments, for example COSY and TOCSY, and these methods also provide valuable information for metabolite identification by correlating different signals from the same molecule. The practicality of investigating plant metabolism with in vivo ^{1}H NMR was first demonstrated in root tissues (36). Recent applications include an analysis of the ligands in barley root exudates (37), the identification of a range of metabolites in the red alga *Gracilariopsis lemaneiformis* (21), and detailed analysis of the composition of tomato fruits (79, 97).

As well as using ^{1}H NMR to detect the signals from hydrogen atoms, it is also possible to use ^{1}H NMR methods to detect the presence of neighboring ^{13}C and ^{15}N atoms. This strategy is known as indirect detection, and it is important partly because it allows the ^{13}C and ^{15}N atoms to be detected with greatly increased sensitivity and partly because it provides further information on the identity of the metabolites. In practice, this is achieved using a further selection of two-dimensional methods (HSQC, HMQC, and HMBC), and these are easier to implement on extracts than in vivo. This approach has been used to analyze anaerobic metabolism in maize root tips (148), the composition of barley root exudates (37), and nitrogenous metabolites extracted from ^{15}N-labeled *Nicotiana plumbaginifolia* cell suspension cultures (93). Indirect detection is also important because it provides a straightforward method for measuring the absolute enrichment of specific carbon and nitrogen atoms with ^{13}C and ^{15}N respectively (e.g. 31).

For carbon the relevant magnetic isotope is ^{13}C. This has a natural abundance of only 1.11%, contributing to a considerably lower sensitivity for ^{13}C NMR than ^{1}H NMR. Accordingly, the application of ^{13}C NMR in unlabeled systems is largely confined to the detection of the most abundant metabolites, such as the organic solutes that accumulate in response to salt stress (6, 132) or certain secondary metabolites (83). As noted above, indirect detection increases the sensitivity of the experiment, and an example of this approach can be found in an analysis of the solutes in sugar beet suspension cells (99). Moreover, the sensitivity of ^{13}C NMR can be greatly increased by labeling the system with exogenously supplied substrates that are either intrinsically permeant or readily transported. The former, with representative examples of their recent application, include acetate (14, 89), carbon dioxide (14), formate (103), and methanol (52), whereas the latter include

amino acids (96, 103, 136) and carbohydrates (31, 33, 100). Again it is often advantageous to use indirect detection methods, although these are not yet widely used.

The nitrogen atom has two magnetic isotopes—^{14}N and ^{15}N—and both can be useful for the detection of metabolites in vivo and in extracts. The practicality of detecting the naturally abundant (99.63%) ^{14}N isotope was first demonstrated in root tissues (18) and subsequently in vivo ^{14}N NMR has mainly been used for the analysis of ammonium and nitrate (48, 63, 74, 75). The low natural abundance of the ^{15}N isotope (0.037%) rules out the detection of unlabeled metabolites, but after labeling with [^{15}N]ammonium or [^{15}N]nitrate it is possible to use in vivo ^{15}N NMR to detect amino acids (62, 63, 131), as well as certain secondary products, such as nicotine, agropine, and conjugated polyamines (39). Indirect detection of ^{15}N-labeled metabolites is potentially advantageous, but its use is largely restricted to tissue extracts because indirect detection of amino-N groups is only possible over a limited range of acidic pH values with the HMQC technique (62, 93, 148).

Phosphorylated metabolites can be identified and quantified in plant material by ^{31}P NMR, and again the practicality of this approach was first demonstrated in root tissues (126). Signals are usually observed from a relatively small number of abundant metabolites—typically phosphomonoesters, inorganic phosphate (Pi), nucleoside triphosphates, and nucleoside diphosphosugars. The metabolic importance of these compounds continues to stimulate many in vivo ^{31}P NMR applications, and the usefulness of such studies can be greatly enhanced by complementary analyses of tissue extracts (7). The phosphorylation of exogenously supplied compounds, such as mannose and galactose (82), choline (19, 91), glycerol (10), and homoserine (9), is also readily observed by in vivo ^{31}P NMR.

It may be useful to conclude this section with a caveat relating to the detection of in vivo NMR signals. It turns out that there are several factors that may make an NMR signal undetectable, even though the ion or metabolite is present at a concentration higher than the detection threshold. First, the signal may be obscured by overlap with larger signals; second, the metabolite may be immobile due to precipitation or tight binding to macromolecules; and third, the signals from a chemically active metabolite may be intrinsically too broad to detect under high-resolution conditions. Although these features of the NMR technique are entirely familiar to NMR practitioners, they can be disconcerting to plant biochemists and they emphasize the need for close collaboration between the two groups.

Metabolite Profiling

Although as yet little used for this purpose, the wealth of metabolic information contained in the NMR spectra of plant tissue extracts suggests that there could be a role for NMR analysis in metabolite profiling. There are at least two reasons for believing that this proposition should be explored further. First, because NMR is a nondestructive technique, it is easy to combine the NMR analysis with a complementary technique, such as GC-MS. The scope of this strategy for analyzing

complex mixtures of plant origin has been clearly demonstrated (34, 36, 37) and further investigation is warranted. Second, the NMR analysis of complex mixtures can be combined with an in-line separation technique, such as liquid chromatography, to create analytical tools that have already been used extensively for metabolic analysis in the field of biomedical research (70, 77).

In contrast to these applications, which demonstrate that numerous specific metabolites can be identified in complex mixtures, other investigators have addressed the question of whether computer-aided comparisons of the ^{1}H NMR spectra of partially fractionated extracts can yield statistically meaningful metabolic fingerprints of the extracted tissue (79, 97). Using this approach, it was possible to show that there were minimal compositional differences between certain transgenic and nontransgenic tomato varieties, but only after accounting for the substantial effects of external factors. Thus up to 30% of the compounds from the fruit of a single line were found to vary significantly between batches grown under nominally the same conditions at different times, and there were also significant variations within a batch (97). This very careful study highlights the formidable challenges that are likely to be encountered in metabolite profiling endeavors as a result of the plasticity of plant metabolism.

DEFINING THE INTRACELLULAR ENVIRONMENT

As well as identifying and quantifying metabolites, it is also important to explore the nature of the intracellular environment in which the interconnecting pathways are operating. This environment is defined by several factors, including ionic composition, energy status, redox state, viscosity, and subdivision through compartmentation. Moreover, changes in the extracellular environment can lead to alterations in one or more of these intracellular parameters. NMR methods, some of which are identical to those already discussed, can be used to probe the intracellular environment, and these applications are discussed in the following paragraphs.

The energetic status of a plant tissue can be assessed quantitatively using ^{31}P NMR (118, 130), and this approach is used routinely to check the physiological state of a sample during in vivo NMR experiments (111). Free ADP levels are generally too low to be detectable by NMR in vivo, so a detectable ADP signal is generally taken to indicate that the respiratory state of the tissue has been compromised. Furthermore, detailed NMR analysis of maize root tip extracts indicates that much of the ADP may be bound to proteins, reducing the chance of detecting it in vivo and complicating the interpretation of the NMR spectra recorded from tissue extracts (59, 60). In passing it may also be noted that NMR imaging has also been used to detect the oxygen diffusion barrier in nitrogen fixing nodules (27, 84).

NMR provides several opportunities for probing the ionic composition of plant tissues (110, 111). Ions that can be observed directly, with the relevant NMR nucleus in parentheses, include potassium (^{39}K), sodium (^{23}Na), chloride

(^{35}Cl and ^{37}Cl) and aluminum (^{27}Al), as well as nitrate (^{14}N and ^{15}N), ammonium (^{14}N and ^{15}N), and phosphate (^{31}P). In other cases, it is possible to use the NMR signal from a suitable ligand to act as a probe for the indirect detection of an ion that binds to it. Thus phosphate can be used as a ^{31}P NMR probe for Mn^{2+} (106), and the ^{133}Cs NMR signals from tissues that have been incubated with cesium salts are sensitive to the intracellular anionic composition (101).

The use of a similar indirect approach to measure intracellular pH is of much greater metabolic significance, and for this purpose NMR-detectable weak acids with pK_a values that are comparable to the pH of the cytoplasm or the vacuole are the most suitable ligands. Inorganic phosphate (^{31}P) and various organic acids (^{1}H, ^{13}C) are the most commonly used endogenous ligands, and the intracellular pH is determined by comparing the position of the in vivo NMR signal with the values obtained from an in vitro calibration procedure (127). NMR is frequently used for pH measurements in plant tissues, and a recent paper on pH changes during the development of cherry tomato fruits provides a representative example (133).

Compartmentation

Although the signals detected by in vivo NMR spectroscopy are averaged over the whole sample, they may still contain interpretable information on the spatial location of the detected metabolites (17). This is possible because differences in the intracellular environment can alter the properties of the NMR signals, leading in favorable cases to information on the compartmentation of the system. Thus, depending on the circumstances, it may be possible to obtain separate signals from subcellular pools, most commonly those originating in the cytoplasm and the vacuole, or to distinguish between signals from inside and outside the cells. The differences in the intracellular environment that give rise to these effects may be intrinsic to the system, with their origin in the spatial variation of the pH, viscosity, ionic composition, or protein content. Alternatively, they may be introduced through the use of paramagnetic ions, in the form of so-called shift reagents or susceptibility reagents. It is also possible to impose externally applied magnetic field gradients in the technique known as NMR imaging, and this powerful method for increasing the spatial information content of the NMR signal is considered in a later section.

Imaging aside, the most commonly encountered manifestation of compartmental analysis involves exploiting the pH difference between the cytoplasm and the vacuole to distinguish the cytoplasmic and vacuolar pools of various ions and metabolites (76, 113). For example, the second pK_a of Pi (6.8) lies between the pH of the cytoplasm (7.4–7.6) and the pH of the vacuole (typically 5–5.5, but often somewhat lower). Since the position of the Pi signal in the ^{31}P NMR spectrum depends on the degree of dissociation of the ion, it follows that separate signals can be detected from the cytoplasmic and vacuolar fractions. Thus the pH dependence of the signal and the pH difference between the two compartments allows

both the subcellular distribution of Pi and the corresponding subcellular pH values to be determined from the same experiment (126). The pH dependence of the NMR signals from a number of other metabolites with suitable pK_a values can also be exploited. These include organic acids (25, 54, 154) and ammonium (75). Moreover, the separate cytoplasmic and vacuolar signals of these metabolites can themselves be used to probe the subcellular compartmentation of particular ions that interact with the observed metabolites. Thus organic acids can be used as pH probes (53) in the same way as phosphate, and both types of ligand can be used to probe the subcellular distribution of paramagnetic cations such as Mn^{2+} (53, 107) and Gd^{3+} (108).

Recently, it has been shown that it is possible to extend these investigations of subcellular compartmentation to amino acids (9, 12). Under normal circumstances, the pK_a values of amino acids are too remote from the cytoplasmic and vacuolar pH values to affect the NMR signals. However, by pretreating certain tissues with an ammonium solution at high pH, it is possible to induce an alkalinization of the cytoplasm that is sufficient to generate separate ^{13}C NMR signals from the cytoplasmic and vacuolar amino acid pools. Although the method has been successfully applied to sycamore cells (9) and the leaves of the Kerguelen cabbage (12), there are some doubts about its generality (47). The method depends on being able to increase the pH of the cytoplasm before the inevitable accumulation of the ammonium in the more acidic vacuole leads to a substantial increase in the vacuolar pH. This suggests that the method is likely to be strongly influenced by such factors as the accessibility of the cells to ammonium, the cytoplasmic to vacuolar volume ratio, and the buffering capacity of the two compartments. However, pH measurements in ammonium-treated maize root tips show that it may be difficult to satisfy these criteria in dense heterogeneous tissues (47). Despite these reservations, this new method can generate new insights into the distribution of amino acids in favorable cases.

There are also a number of instances in which subcellular information can be obtained from plant tissues on the basis of intrinsic factors other than pH. The first example is the chloride ion, where interactions with cytosolic proteins render the cytoplasmic pool invisible and allow the vacuolar pool to be monitored in vivo directly (150). Second, separate signals from cytoplasmic and vacuolar cesium are readily observed when plant tissues are incubated with cesium salts because the ^{133}Cs NMR signals are highly sensitive to the anionic composition and protein content of each compartment (101). Thus ^{133}Cs NMR provides opportunities for probing cation transport and the compartmentation of anions. Third, bound manganese in chloroplasts gives rise to an internal magnetic susceptibility effect that separates the ^{1}H NMR signals from chloroplast and nonchloroplast water in the leaves of several species, and this permits measurements of water exchange across the chloroplast membrane (90).

Bulk magnetic susceptibility effects have also been exploited in another recent development in the application of NMR to compartmental analysis (145, 149). It has been shown that adding nonpermeant and nontoxic complexes of paramagnetic

ions to the suspending medium provides a versatile method for distinguishing all intracellular and extracellular signals from samples with cylindrical symmetry. The method has been applied to roots, shoots, and giant algal cells, and it is likely to find particular application in the measurement of fluxes across the plasma membrane (145).

EXPLORING PATHWAYS AND THEIR OPERATION

The primary motivation for using NMR methods to investigate plant metabolism stems from the current incomplete understanding of the way in which individual pathways operate within metabolic networks. Thus any method that can probe the operation of pathways in vivo is potentially valuable, and as this section and the one that follows will make clear, NMR makes a significant contribution to this endeavor. There are, of course, many other ways of investigating metabolic pathways and so NMR is but one of many complementary approaches. At the same time, an NMR analysis can often provide a very direct and convenient window on metabolism, and this is particularly true in the burgeoning field of stable isotope labeling (120).

Ultimately, the ability to explore the operation of metabolic pathways with NMR is simply an extension of its use as a nondestructive technique for identifying metabolites in tissues and extracts. Thus much can be achieved by using in vivo NMR methods to measure the changes in detectable metabolites as a function of time in labeled and unlabeled tissues. The resulting in vivo time courses have an inherent statistical advantage over time courses that have been constructed from a series of replicate measurements because all the spectra are recorded from the same sample. Moreover, in vivo time courses facilitate the correlation of simultaneous metabolic events, because the metabolic information is recorded throughout the experiment rather than at arbitrary time points, and they also make it easier to characterize oscillatory phenomena (159). These benefits can only be obtained by paying particular attention to the physiology of the tissue in the magnet (7, 111, 113, 129), and in some cases it may be advantageous to forgo them in favor of the greater sensitivity and resolution of an extract analysis. For example, there is no particular advantage in using in vivo NMR methods for the analysis of steady-state isotopic labeling experiments, and the analysis of a tissue extract is usually much more informative (138).

NMR methods are particularly suitable for the analysis of labeling experiments (80). The information that can be obtained includes both the relative and absolute fractional enrichments of particular atoms, as well as the identity and relative abundance of the isotopomers in the extract. Isotopomers are sets of labeled molecules that contain different distributions of labeled atoms, for example a mixture of [1,2-^{13}C]- and [1,3-^{13}C]glucose. NMR has a particular advantage over GC-MS, which is the other principal method for analyzing stable isotope labeling, because it provides direct information on the positional distribution of the labeled atoms

within a molecule. This arises because structurally inequivalent carbon atoms within the same metabolite are usually magnetically inequivalent as well, and therefore give separate ^{13}C NMR signals. Furthermore, the presence of neighboring magnetically inequivalent carbon atoms causes a characteristic splitting of the NMR signals. As a result, the signals from [1,2-^{13}C]- and [1,3-^{13}C]glucose in the mixture of the two isotopomers above are readily identified. Some of the first metabolic applications of isotopomeric analysis were in plant metabolism (140, 141) and these early papers demonstrated the value of the approach in identifying and quantifying the pathways responsible for the redistribution of the label.

The in vivo and in vitro approaches to pathway analysis sketched out above become even more informative when combined with the controlled manipulation of the system. Transgenic technology offers unprecedented opportunities for probing metabolic pathways (20), and NMR has a role to play in defining the metabolic phenotypes of transgenic organisms (69). The first NMR investigations of transgenic plants are beginning to appear (38, 158) and this is likely to be an increasingly important feature of the field. At the same time, traditional approaches to perturbing metabolism, including the use of inhibitors (104, 163) and manipulating the intracellular pH (24, 45), continue to provide opportunities for testing metabolic hypotheses in vivo. It is against this background that we review the areas of plant metabolism in which NMR is making a significant contribution.

Photosynthetic Carbon Metabolism

NMR investigations of the pathways of photosynthetic carbon metabolism usually depend on the analysis of tissue extracts because of the apparently insurmountable difficulties in applying in vivo NMR methods to illuminated leaves (111). Following an early application of ^{13}C NMR to the analysis of photorespiration in ^{13}C-labeled C3 and C4 plants (140), the interest in using NMR methods was largely confined to the empirical analysis of site-specific natural isotope fractionation (SNIF) in the end-products of photosynthetic carbon metabolism (88). SNIF involves measuring the fractional enrichment or depletion of particular carbon and hydrogen atoms, and this can often be done conveniently by ^{13}C and ^{2}H NMR. Thus in one application, a ^{2}H SNIF-NMR analysis showed that it was possible to distinguish between C3 and C4 plants on the basis of the deuterium distribution in glucose samples derived from hydrolyzed starch (164). However, the emphasis was on establishing an empirical correlation, rather than on probing the metabolic implications of the results, and this aspect was not examined in detail.

More recently, two studies have shown that it is possible to extract mechanistic information from the nonrandom distribution of deuterium in the glucose extracted from sucrose (143) and leaf starch (142). First, in an important study on carbon transport, it was shown that the bulk of the carbon that is exported from chloroplasts at night is in the form of hexoses rather than triose phosphate (143). This conclusion was based on a ^{2}H NMR analysis of the glucose moiety of the sucrose obtained from leaves labeled with ^{2}H-enriched water, and this analysis showed that the bulk

of the sucrose must have been synthesized directly from glucose. In a subsequent study, it was found that the glucose isolated from leaf starch was depleted in deuterium at carbon 2 in comparison with the glucose extracted from leaf sucrose or endosperm starch. This was explained in terms of a kinetic isotope effect that manifested itself because of the disequilibrium of the chloroplast phosphoglucose isomerase (142). It remains to be seen whether the novel approach used in these studies can be extended to the analysis of other pathways that include solvent exchange steps.

Nonphotosynthetic Carbon Metabolism

Several substrates can be used to introduce ^{13}C-labels into the pathways of primary metabolism in heterotrophic plant tissues. The use of [^{13}C]bicarbonate, which provides a convenient method for investigating the synthesis of malate and citrate (25, 54), is one possibility, but it is the use of [^{13}C]glucose that is currently attracting most attention. An important early study highlighted the reversible interconversion of hexose and triose phosphates in the cytosol of wheat endosperm (65). This paper also provided strong evidence against the import of triose phosphates into the amyloplast for the synthesis of starch, and thus played a significant part in focusing attention on the uptake of hexose phosphates. Cycling between hexose and triose phosphates has since been studied in a number of other systems, including potato tubers (160) and potato cell suspensions (71), and in a recent study in transgenic tobacco cells it was shown that elevated levels of fructose-2,6-bisphosphate (Fru-2,6-P_2) increased the rate of cycling (38). In the latter study the analysis of the cycling rate provided compelling evidence that pyrophosphate: fructose-6-phosphate 1-phosphotransferase (PFP) is not necessarily fully activated in vivo in heterotrophic tissue and that its activity can be modulated by changes in the level of Fru-2,6-P_2 within the normal physiological range (38).

The usefulness of [^{13}C]glucose labeling extends far beyond the exploration of the first few steps in the glycolytic pathway. In an important paper (31), it was shown that glycolysis, the oxidative pentose phosphate cycle, sucrose turnover, polysaccharide synthesis, and entry into the TCA cycle could all be detected in maize root tips by using suitable isotopomers of [^{13}C]glucose and analyzing the specific enrichment of the carbons in a range of carbohydrates and amino acids. Most of the 20 metabolic fluxes that were quantified in this study were determined from the NMR data, providing an impressive demonstration of the potential of ^{13}C NMR in the analysis of the pathways of intermediary metabolism. The pathways that are operating can be determined by identifying the labeling of particular intermediates and end-products. For example, the relative contribution of malic enzyme and pyruvate kinase to the synthesis of pyruvate has been assessed in maize root tips by measuring the incorporation of label from [1-^{13}C]glucose into alanine (31, 33). In this case synthesis via pyruvate kinase leads to labeling of carbon 3, whereas synthesis via malic enzyme leads to labeling at all three alanine carbons. Thus the labeling of carbon 2 provides clear evidence that the malic enzyme

pathway is active (125), and a quantitative analysis of the alanine isotopomers allows an assessment of the contribution of the pathway to respiration (31, 33). The oxidative pentose phosphate pathway is another pathway that is accessible via [^{13}C]glucose labeling (31), and the suitability of using this approach for analyzing the subcellular compartmentation of the pathway is under investigation (72, 73, 138).

Valuable information about the operation of certain metabolic pathways can also be obtained by a careful ^{31}P NMR analysis of the levels of phosphorylated metabolites (105). Thus NMR analysis of the glycolytic intermediates, ATP, NAD^+, $NADP^+$, and NADPH, in perchloric acid extracts, provided strong evidence for an activation of glycolysis and the oxidative pentose phosphate pathway as part of the initial response of tobacco cells to the fungal elicitor cryptogein (105). As in many other NMR studies, perturbing the system, in this case by applying the fungal elicitor, shed light on the metabolic response of the cell suspension. Other commonly used perturbations include sucrose starvation (11, 30), oxygen deprivation (33), and salt stress (136).

Fermentation Pathways

Oxygen deprivation causes serious disruption of the normal aerobic pathways of plant metabolism (32). This disruption has been studied extensively by NMR and comprehensive reviews of the field have been published elsewhere (114, 130). In outline, the NMR studies of the anoxic response have focused on the contribution of particular pathways to energy production and pH control in the absence of oxygen, and on the origin and significance of the pH changes that occur in hypoxic and anoxic tissues.

Initially, in vivo NMR methods were used to show that (*a*) the switch from lactate to ethanol production observed under anoxia in maize root tips was caused by a fall in the cytoplasmic pH (124); and (*b*) cytoplasmic acidosis, caused by inadequate pH regulation, could be used as an indicator of flooding intolerance (121, 123). Subsequently, the analysis was extended to allow an assessment of the contribution of malate and amino acid metabolism to intracellular pH regulation under anoxia, and it was concluded that (*a*) the initial acidification of the cytoplasm could be attributed to lactate and alanine synthesis; (*b*) this initial fall in pH was limited by the activation of pyruvate decarboxylase and malic enzyme; and (*c*) the proton-consuming synthesis of GABA was quantitatively insignificant during the initial stabilization of the pH (125). More recent NMR investigations of the metabolic response to oxygen deprivation include in vivo demonstrations of the pH-sensitivity of pyruvate decarboxylase (45) and glutamate decarboxylase (GDC) (24) and an analysis of the metabolic factors that confer improved pH regulation on root tips that have been acclimated to a low oxygen environment (161–163). While there is continuing debate about the extent to which the picture that emerges from these studies is generally applicable (112, 115, 139), it is clear that it constitutes a major contribution to our understanding of plant anaerobiosis.

One-Carbon Metabolism

In yeast it has been possible to make significant progress in the analysis of one-carbon metabolism by using ^{13}C NMR to measure metabolic fluxes in genetically modified cells (5). Encouragingly, ^{13}C NMR has recently been adopted in the study of one-carbon metabolism in plants, and the metabolism of serine and glycine via glycine decarboxylase (GDC) and serine hydroxymethyltransferase (SHMT) has been explored in both photosynthetic (103, 104) and nonphotosynthetic (96) cells. These experiments employ ^{13}C-labeled substrates, and their interpretation depends on the subsequent quantitative analysis of the [2-^{13}C]-, [3-^{13}C]-, and [2,3-^{13}C]serine isotopomers. This approach has generated new insights into serine and glycine metabolism, and recent evidence suggests that the mitochondrial 5,10-methylene-tetrahydrofolate (CH_2-THF) pool does not equilibrate with the cytosolic and plastidic pools (96, 104). Methanol also enters metabolism via the THF pool and this has been investigated using [^{13}C]methanol and ^{13}C NMR (52). NMR applications to other parts of one-carbon metabolism include analyses of the uptake of phosphocholine and phosphoethanolamine (7), and the testing of several assumptions and predictions of a model of choline metabolism in transgenic tobacco (91). The use of genetic engineering and flux analysis is likely to be the key to understanding the regulation of one-carbon metabolism in plants (57), and the examples given here suggest that NMR analysis will play a significant role in achieving this objective.

Nitrogen Metabolism

Good evidence for the assimilation of ammonium via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle has been obtained by in vivo ^{15}N NMR in many systems, including cell suspensions (4, 131), embryogenic cultures (62, 63), root tissues (3, 40), and the peat moss *Sphagnum fallax* (64). The label is usually introduced in the form of [^{15}N]ammonium or [^{15}N]nitrate, and the identification of the pathway is based on an analysis of the subsequent incorporation of the label into amino acids in the presence and absence of inhibitors of GS and GOGAT. Particular interest attaches to the potential role of glutamate dehydrogenase (GDH) in the assimilation process, but with the exception of some observations on *Chlorella fusca* (2, 23), all the NMR analyses are consistent with a negligible role for GDH in the synthesis of glutamate. In contrast, the combined use of in vivo ^{15}N NMR and GC-MS has provided good evidence for the involvement of GDH in the oxidative deamination of glutamate in sucrose-starved carrot cells (46, 131). This conclusion has been queried (98), but a recent ^{13}C NMR investigation of isolated mitochondria has provided further support for the catabolic role of GDH (8). NMR evidence has also been presented for the involvement of the GS/GOGAT cycle in the recycling of the ammonium released by the deamination of phenylalanine during phenylpropanoid metabolism (116, 152).

Glutamate and glutamine are the precursors of many other metabolites, and in vivo ^{15}N NMR has been used to investigate several other pathways by following

the subsequent metabolism of the ^{15}N-label. One example is the conversion of glutamate to GABA by glutamate decarboxylase (GDC), which was shown to be stimulated by processes that acidify the cytoplasm in both carrot cells (24) and root cultures of *Datura stramonium* (40). This result was consistent with the pH-dependent kinetic properties of GDC, and it suggests a role for GABA in pH regulation (24, 29) since GDC catalyzes a proton-consuming reaction. Elsewhere the operation of the ornithine cycle has been inferred on the basis of the substantial labeling of arginine in some tissues (1, 62, 63), while in other studies, it has been possible to investigate pathways of secondary metabolism (41–43). In one study, in vivo ^{15}N NMR observations of the conjugated polyamine pool in a *D. stramonium* root culture led to the hypothesis that the phytohormone-induced dedifferentiation of the culture required the presence of free polyamines. The hypothesis was supported by results obtained with metabolic inhibitors (42).

Phosphorylation

In vivo ^{31}P NMR allows the ready detection of phosphorylated metabolites present at tissue concentrations of the order of 1 μmol g^{-1} fr wt and this permits the investigation of a range of phosphorylation reactions. In one approach, plant tissues are supplied with a precursor that is readily phosphorylated, and NMR is used to monitor the accumulation, compartmentation, and subsequent metabolism of the product. Early applications examined the metabolism of galactose (82) and choline (19), and more recently, there have been detailed investigations of the phosphorylation of glycerol (10) and homoserine (9). For example, supplying glycerol to sucrose-starved sycamore cells led to the rapid accumulation of glycerol-3-phosphate in the cytoplasm (10). This led to an arrest of gluconeogenesis and the oxidative pentose phosphate pathway, and although glycerol prevented the development of autophagy, most probably by providing carbon skeletons for respiration, it was unable to support biosynthesis. Glycerol also caused the accumulation of a cytoplasmic pool of *O*-phosphohomoserine in cells supplied with sucrose, and this was interpreted in terms of an inhibitory effect of glycerol-3-phosphate on the pathways utilizing *O*-phosphohomoserine (10).

Several other approaches have been used in recent investigations of phosphorylation reactions. First, a bacterial polyphosphate kinase was expressed in the chloroplasts of potato plants with the intention of creating a novel Pi reserve for photosynthesis. The subsequent NMR analysis provided an unequivocal demonstration of the existence of a polyphosphate pool in the transgenic plants and a quantitative measure of its structural organization (158). Second, nucleotide metabolism has been investigated in isolated potato mitochondria by combining ^{31}P NMR measurements of phosphotransferase activity with oxygen electrode measurements of the respiratory state. This novel approach highlighted the interaction between adenylate kinase and ATP synthase in the regeneration of ATP from AMP and nucleoside diphosphates (122). Finally, the steady-state fluxes between a number of bioenergetically important phosphorylated metabolites have

been measured by ^{31}P NMR (137) and this is discussed in the section on analyzing metabolic flux.

Mycorrhizal Metabolism

The challenge of observing fungal and plant metabolic processes separately in intimately connected symbionts is considerable (153). However, the identification of NMR signals from metabolites unique to either the host or the fungus in spectra recorded from the intact system provides a method for probing mycorrhizal metabolism (102). Thus the uptake and transfer of phosphorus by the fungal partner, which is the source of the principal nutritional benefit to the plant, can be analyzed by ^{31}P NMR. However, from a mycocentric perspective, the crucial transfer of fixed carbon from the plant to the fungus can be analyzed by ^{13}C NMR.

The uptake and metabolism of Pi by ecto- and endomycorrhizal fungi in the free-living and symbiotic states, as well as the structure and metabolism of polyphosphates, continues to be the subject of investigations by ^{31}P NMR (50, 102, 109). The chelation of aluminum (87) by polyphosphates and the effects of aluminum on phosphate uptake and metabolism (49) have also been assessed. ^{31}P NMR has also been used to analyze phosphate and phosphonate levels in the roots and shoots of *Allium cepa* treated with a fungicide (155).

Valuable information on the pathways of carbon metabolism that operate at different stages of the fungal life cycle has been obtained by ^{13}C NMR. These studies have provided evidence as to which forms of carbon are taken up by the fungus (100, 102), as well as identifying fungal storage compounds (16, 86, 147), and they have also led to the delineation of the principal pathways of fungal carbon metabolism (14, 86, 100). Interestingly, experiments on an endomycorrhizal symbiosis have revealed that hexose acquired by the fungus inside the root is converted to lipid before being exported to the extraradical mycelium, where some of it is converted back into carbohydrate for anabolic purposes (100). Taken together, these studies have significantly advanced our knowledge of carbon metabolism in ecto- and endomycorrhizas (15, 56).

ANALYZING METABOLIC FLUX

As described in the preceding section, NMR methods can be used to investigate numerous metabolic pathways either in vivo or via the analysis of tissue extracts. Although the qualitative observation of flow through a pathway can be informative, an important trend in recent years has been to use NMR techniques to obtain quantitative measurements of metabolic flux. Currently, there is particular interest in calculating such fluxes from the measurements of fractional enrichment obtained in labeling experiments. Comparisons can then be made between the fluxes obtained from different systems, or from the same system in different physiological states, and this can lead to insights into the integration and regulation of

metabolism. Ultimately, the goal of such an approach is a predictive model of the metabolic network, in which the observed fluxes can be reconciled with the kinetics properties of the enzymes. The increasing emphasis on quantitative NMR analyses of metabolic pathways is a significant development in the plant NMR field, and several features of the NMR analysis of metabolic flux are discussed here.

One approach to flux measurement is to construct a time course on the basis of a series of in vivo or in vitro NMR spectra. The accumulation of phosphorylated metabolites, for example glycerol-3-phosphate (10), or labeled end-products, for example a methyl glucoside (52), in cells supplied with suitable precursors are typical situations in which metabolic time courses can be obtained, and net fluxes to such compounds, if not data on turnover and lifetime, are often reported. However, as discussed elsewhere (138), a more flexible approach to the measurement of metabolic flux is to introduce suitable labels into the metabolic network and then to analyze the distribution of the label after the system has reached an isotopic steady state. This approach exploits the underlying relationship between the observed labeling of a metabolite and the multiple pathways that might lead to labeling in a complex metabolic network. One immediate advantage of the steady state approach is that it is only necessary to record a limited number of NMR spectra, each with a high information content, while another advantage is that NMR is ideal for discerning the fractional enrichment at specific carbon atoms in particular metabolites. These fractional enrichments provide primary data from which metabolic fluxes can be deduced, provided it is possible to construct an appropriate flux model that matches the underlying metabolic network.

A specific example that illustrates the approach is the redistribution of ^{13}C-label from carbon 1 to carbon 6 in hexose phosphates. This redistribution is caused by cycling between the hexose phosphate and triose phosphate pools, and its extent is determined by the other processes that contribute to the labeling of the hexose phosphate pool. This scrambling of the label has been investigated in several systems (38, 65, 71, 160) and a detailed theoretical analysis has been presented (138). Qualitatively, if the gluconeogenic flux is small relative to the flux into the hexose phosphate pool via hexokinase, then little label will reach carbon 6, whereas if the reverse is the case, and cycling is rapid, then equilibration will be complete and carbons 1 and 6 will have the same fractional enrichment. In fact, the observed situation is usually intermediate, with ratios of the fractional enrichment at carbon 6 to carbon 1 in the range 15–30% (38). This shows that the degree of cycling is significant, and a quantitative analysis of the fractional enrichments gives a quantitative measure of the ratio of the two fluxes of label into the hexose phosphate pool (138). The principal assumptions in such an analysis are that the pool sizes are constant, that the labeling has reached steady state, and that no complicating pathways are operating to alter the redistribution of the label. Note also that the analysis of the fractional enrichments generates a flux ratio, rather than absolute fluxes through the contributing pathways, and that no metabolite pool

sizes or kinetic parameters for the enzymes that generate the fluxes are required or predicted.

In principle, the analysis of fractional enrichments can be used to investigate other competing pathways (33), and given the necessary data, multiple fluxes can be obtained from much more extensive metabolic networks (31). However, there have been few investigations of plant metabolism on this scale, and even when the necessary labeling data have been obtained for smaller-scale flux analyses, few attempts have been made to extract the metabolic fluxes. For example, in a study of carbon metabolism in a mycorrhizal fungus, prolonged exposure to a range of ^{13}C-labeled precursors allowed the measurement of the fractional enrichment at every carbon atom in trehalose (14). However, the analysis was restricted to identifying the pathways of carbon flow from the labeling pattern, even though the converging pathways involved should permit a steady-state flux analysis. The emphasis in this and many other studies is on pathway delineation, and exploring the possibility of reaching an isotopic steady state with the aim of quantifying relative metabolic fluxes is usually a low priority. The development of an appropriate flux model for converting fractional enrichments into the underlying metabolic fluxes is often not trivial, and this may have a deterrent effect on the application of the approach to plant metabolism. Indeed, the theoretical development of modeling approaches in general appears to be outstripping their adoption by the plant science community (51).

There is another way in which NMR can be used to measure metabolic fluxes from a steady-state analysis. This approach can shed light on unidirectional fluxes that occur over a time scale of seconds between certain phosphorylated metabolites. In this type of experiment the labels are magnetic and the fluxes are revealed through the use of ^{31}P NMR. In the simplest version of this technique, individual fluxes are measured between pairs of phosphorylated metabolites, notably ATP and Pi (128). The same approach has also been used to investigate the flux between UDPglucose and glucose 1-phosphate in maize root tips (119). An alternative version of the same experiment (EXSY) allows the simultaneous detection of all the observable fluxes (137). In practice, the experiment is lengthy and somewhat demanding, but it allows the observation of a number of important metabolic steps. The power of the method has clearly been demonstrated (137), and it awaits application in appropriate situations.

Quantifying fluxes is not an end in itself and it is comparisons between fluxes that leads to metabolic insights. Thus flux ratios or differences between fluxes within a metabolic network are likely to be useful, and comparing the same fluxes in closely related systems can also be informative. A good example can be found in the study of the contribution of malic enzyme to the synthesis of pyruvate in maize root tips where it was shown that the activity of malic enzyme increased by more than a factor of six early in hypoxia (33). Another approach is to use genetic manipulation to alter the metabolic landscape, as in an investigation of the effect of Fru-2,6-P_2 on the activity of PFP and hexose phosphate to triose phosphate cycling (38). Information obtained by manipulating metabolic fluxes is likely to

be crucial in the construction of the metabolic models needed for an accurate and detailed description of metabolic networks.

IMAGING METABOLITES

As indicated above, NMR imaging provides a method for extracting spatial information from NMR signals. The method is most easily applied to the ^{1}H NMR water signal, since this is the strongest signal that can be detected in vivo, and the images reflect the state and distribution of the water in the imaged tissue (26, 85, 111). The images can be interpreted in terms of tissue anatomy and water movement, and the technique has now reached the point where it is capable of generating physiologically important information. The current power of NMR imaging can be judged from a recent paper on water flow in castor bean seedlings (66). Water flow through the xylem and phloem was quantified in intact seedlings using a flow-sensitive NMR imaging method, and it was shown that conventional measurements of the flow through the phloem, based on exudation rates, greatly overestimated the actual flow rate. It was also shown that there is an internal circulation between the phloem and the xylem, and that this phenomenon maintains water flow through the xylem in the absence of transpiration. Overall this paper provides a powerful demonstration of the contribution to be made by NMR imaging in studies of water flow (66).

Although the metabolites in a tissue are invariably present at concentrations that are orders of magnitude lower than the tissue water, there are have been several reports in which imaging methods were used to generate maps of the more abundant primary and secondary metabolites (61, 68, 92, 94, 95, 156, 165). The aim of this approach is to develop methods for mapping the tissue distribution of carbohydrates and amino acids, and from a metabolic perspective it is the spatial resolution of such maps that will eventually determine their value. However, it is still too early to define the likely limits of metabolic imaging and there have been few comparative studies of the different techniques (156).

In broad terms, there has to be a compromise between the spatial and temporal resolution of the experiment, and the current status of the field can be judged from some representative results (94, 159, 165). Thus sucrose, glucose, glutamine/glutamate, lysine, and arginine have been simultaneously mapped in the hypocotyls of castor bean seedlings using the technique known as correlation peak imaging (94, 165). The sensitivity of this experiment was judged to be sufficient to allow the detection of a metabolite present at 10 mM with a spatial resolution of 0.375 by 0.375 by 4 mm (a voxel volume of 0.56 μl) in 4 h 33 min (94). Sucrose has also been mapped on its own in the castor bean hypocotyl using the technique known as chemical shift imaging, and in this case a spatial resolution of 0.094 by 0.094 by 2 mm (a voxel volume of 0.018 μl) was achieved in 1 h 10 min (159). Although this approach to in vivo histochemistry is still limited by the available sensitivity, chemical shift imaging has

generated some physiologically important information about the translocation of sucrose in the phloem (159). In particular, it was shown that sucrose equilibration occurred between vascular bundles, indicating radial translocation through the parenchyma.

Arguably the most exciting recent technical development in metabolic imaging is the localization of ^{13}C labeling (58). This can be achieved by indirect ^{1}H detection of the ^{13}C-labeled metabolites and it is a technically demanding area still in development. In the only study so far, ^{13}C-labeled glucose and fructose were taken up by the cotyledons of castor bean seedlings, and the subsequent synthesis of labeled sucrose and its translocation to the hypocotyl were observed with a time resolution of the order of 1 h. The spatial resolution achieved in this study was markedly less than in the chemical shift–imaging study (159), but the ability to measure newly synthesized sucrose by monitoring the incorporation of a ^{13}C label is a potential advantage. It remains to be seen whether this will become the basis of a worthwhile metabolic tool, but the current pace of development in the imaging field gives grounds for optimism.

CONCLUDING REMARKS

Modern metabolic research is increasingly aimed at defining metabolic phenotypes. This is not merely an exercise in identifying and quantifying metabolites, otherwise known as metabolite profiling, or even of mapping their distribution, since defining a metabolic phenotype also involves gaining an understanding of the integration and regulation of the underlying metabolic transformations. This in turn depends on delineating pathways, defining the intracellular environment, and quantifying metabolic fluxes. As discussed, NMR has a part to play in defining metabolic phenotypes, and its value is further increased when it is used in conjunction with the other techniques of metabolic analysis. Against this background it is arguable that NMR is likely to become increasingly important in two areas. First, there is considerable potential for using NMR methods in the characterization of transgenics, and indeed for using transgenic material to make NMR experiments more informative. Second, it is clear that the combination of steady-state isotope labeling and NMR detection of the resulting fractional enrichments can provide valuable information for metabolic flux analysis. Thus one may reasonably expect that NMR will continue to make worthwhile contributions to our understanding of plant metabolism.

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