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Review

Advances of high-resolution NMR techniques in the structural and metabolic analysis of plant biochemistry

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Abstract

Rapid progress in instrumentation and software made nuclear magnetic resonance spectroscopy (NMR) one of the most powerful analytical methods in biological sciences. Whereas the development of multidimensional NMR pulse sequences is an ongoing process, a small subset of two-dimensional NMR experiments is typically sufficient for the rapid structure determination of small metabolites. The use of sophisticated three- and four-dimensional NMR experiments enables the determination of the three-dimensional structures of proteins with a molecular weight up to 100 kDa, and solution structures of more than 100 plant proteins have been established by NMR spectroscopy. NMR has also been introduced to the emerging field of metabolomics where it can provide unbiased information about metabolite profiles of plant extracts. In recent times, high-resolution NMR has become a key technology for the elucidation of biosynthetic pathways and metabolite flux via quantitative assessment of multiple isotopologues. This review summarizes some of the recent advances of high-resolution NMR spectroscopy in the field of plant sciences.

Keywords: NMR spectroscopy; Metabolomics; Metabonomics; Biosynthesis

Contents

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1. Physical principles

Atomic nuclei with non-zero spin adopt quantized orientations in magnetic fields (for details about the physical principles of NMR, see Abragam, 1961; Ernst et al., 1987). Transitions between these quantized states can be induced by radiofrequency irradiation. The transition energies are modulated by the nature of the atom (i.e. ^{1}H, ^{13}C, ^{15}N, etc.) as well as by the electronic environment of each respective nucleus which is the origin of the different chemical shift values of atoms occurring in different molecular positions.

Since the energy differences between the quantized orientations of individual nuclei are small (in the range of μeV), the differences in the occupancy of the respective higher and lower level of a given nuclear species are small. This is the main reason for the relatively low sensitivity of NMR spectroscopy; even with the most advanced instrumentation, at least μmol concentrations of a given analyte are required. By comparison with other bioanalytical methods such as mass spectroscopy, gas chromatography, high-performance liquid chromatography and capillary electrophoresis, the sensitivity of NMR analysis is lower by orders of magnitude. However, this relatively low sensitivity is in part compensated by the abundance of molecular information that can be obtained from typical NMR spectra.

Most of the nuclides which are constituents of bioorganic molecules have non-zero nuclear spin. Specifically, the nuclei of ^{1}H, ^{3}H, ^{13}C and ^{15}N and ^{31}P are all characterized by spin 1/2 and afford NMR signals with narrow line width that are best suited for biological studies (the use of ^{3}H is severely restricted by its radioactivity requiring special precautions). ^{2}H and ^{14}N have spin 1 and afford broad NMR signals that are generally less favourable for NMR studies of biological materials; nevertheless, ^{2}H can be especially useful for certain mechanistic applications and for the measurements of $^{1}H/^{2}H$ isotope ratios revealing the geographical or biosynthetic origin of natural samples (Robins et al., 2003; Schmidt et al., 2003).

The abundant ^{12}C isotope has a gg nucleus with even (and identical) numbers of protons and neutrons, and its resultant nuclear spin is 0 (zero). Hence, the vast majority (98.9%) of the carbon present in natural organic matter is not available for NMR detection. This is one of the reasons for the low sensitivity of ^{13}C NMR of biomolecules. The limitation can be sometimes overcome by artificial ^{13}C labelling, a common approach for the study of biological macromolecules. On the other hand, the low abundance of ^{13}C in natural organic matter enables a very wide variety of dynamic studies of metabolism where a deviation from the natural ^{13}C abundance is artificially generated (see below).

The resonance frequency ν_0 (Hz) for a given atom type in a given magnetic field is described by the gyromagnetic ratio in the following equation:

$$\nu_0 = \gamma B_0/2\pi \quad (1)$$

where γ is the gyromagnetic ratio and B_0 is the magnetic field strength.

The detection sensitivity for nuclides with spin 1/2 is described by the following equation:

$$S/N \approx n\gamma_e\sqrt{\gamma_d^3 B_0^3 t} \quad (2)$$

where S/N is the signal-to-noise ratio, n the number of nuclear spins being observed, γ_e the gyromagnetic ratio of the spin being excited, γ_d the gyromagnetic ration of the spin being detected, B_0 the magnetic field strength, and t is the acquisition time.

Tritium (^{3}H) is the most sensitive nucleus for NMR detection, closely followed by ^{1}H and ^{19}F, whereas the sensitivities for ^{13}C, ^{31}P and ^{15}N are lower by orders of magnitude. Little use, however, can be made of the excellent NMR sensitivity for ^{3}H where measurements are limited to artificially ^{3}H enriched material and require protection of the spectroscopist from radioactivity. Physical parameters of biologically important nuclides are summarized in Table 1.

2. Instrumentation

NMR technology has passed through a phase of very rapid development. The first generation of commercial NMR instruments that became available in the 1950s operated at a ^{1}H resonance frequency of 60 MHz and was equipped with electromagnets or permanent magnets. A

Table 1
NMR data for important nuclei in phytochemistry

Isotope	Spin	Natural abundance %	Relative sensitivity	Absolute sensitivity
^{1}H	1/2	99.985	100.0	100.000000
^{2}H	1	0.015	1.0	0.000145
^{3}H	1/2	3×10^{-16}	121.0	4×10^{-16}
^{13}C	1/2	1.100	1.6	0.017600
^{14}N	1	99.634	0.1	0.101000
^{15}N	1/2	0.366	0.1	0.000385
^{19}F	1/2	100.000	83.3	83.300000
^{29}Si	1/2	4.670	0.8	0.036900
^{31}P	1/2	100.000	6.6	6.600000
^{77}Se	1/2	7.600	0.7	0.052500

The absolute sensitivity (normalized to the ^{1}H NMR sensitivity = 100) accounts for the natural abundance of the respective isotope.

major breakthrough in the early 1970s was the introduction of superconducting magnet coils operating at liquid helium temperature. At present, the limit for commercially available high-field instruments is at a field strength of 22.3 T equivalent to a ^{1}H resonance frequency of 950 MHz. The escalation of field strength serves both the improvement of spectral resolution and detection sensitivity.

A second major breakthrough was the development of pulsed broad band radiofrequency excitation of the sample that replaced the earlier, monochromatic continuous wave technology within a few years (Ernst and Anderson, 1966). The readout resulting from pulsed broadband excitation (the so-called free induction decay, FID) must be converted into a conventional spectrum by Fourier transformation (FT), and progress in computer technology was therefore essential for the further development of NMR instrumentation. The introduction of FT-NMR spectroscopy was honoured by the award of a Nobel Prize to Richard Ernst in 1991 (“for his contributions to the development of the methodology of high-resolution nuclear magnetic resonance spectroscopy”).

Whereas specialized computer processors were initially used for Fourier transformation, primary data from NMR are now universally processed with standard hardware. In fact, standard office computers in conjunction with low-cost NMR software are now in general use for the purpose.

Autosamplers that mechanically change NMR tubes by a preset program in conjunction with automatically tuneable probeheads went a long way to facilitate the handling of large sample numbers. More recently, flow-through cells enable the almost-real-time analysis of a series of fractions from a high-performance chromatography column or the analysis of large sample arrays in rapid sequence (Wolfender et al., 2005).

A recent advance in the ongoing battle for improved sensitivity of even the most advanced NMR spectrometers was the development of microcoil probeheads where the sample volume can be reduced by a factor of 20–100 as compared to conventional probe heads (Aramini et al., 2007). A further milestone in sensitivity improvement was the introduction of cryogenically-cooled probes where the radiofrequency receiver coil and the preamplifiers operate at very low temperatures (ca. 25 K) in order to reduce the level of thermal noise (Styles et al., 1984; Kovacs et al., 2005).

3. Experimental techniques

The NMR analysis of a chemical structure (including constitution/connectivity, configuration and conformation) is based on (i) the (very slight) modulation of the resonance energy by the electronic environment of a given atomic nucleus resulting in chemical shift dispersion and (ii) the mutual influence that neighbouring nuclei exert on each other via chemical bonds (scalar coupling) and/or by interaction through space (nuclear Overhauser effect, NOE). Whereas early NMR structure analysis was predominantly based on the interpretation of chemical shift data, coupling analysis has become progressively more important as a result of the explosive development of methods for the analysis of spin networks. This is explained in more detail below.

Hydrogen occurs in nature almost exclusively as the ^{1}H isotope. These nuclei have spin 1/2 and interact through bonds and through space. The strength of interaction through bonds depends on the number and types of bonds but also on conformational aspects and is observed as fine structure in the signals which may appear as doublets or multiplets depending on the topology of the network. ^{1}H–^{1}H coupling constants in organic molecules can have values up to 20 Hz, with typical values between 5 and 10 Hz. Coupling can be detected if the coupling constant is at least in the range of the natural line width of ^{1}H. In practical terms, coupling with coupling constants above 1 Hz can typically be detected with routine spectrometers. That limits the range of observable coupling between protons to a maximum distance of three scalar bonds in many molecules, although couplings through four or even five bonds are not uncommon. The influence of the dihedral angle of protons connected by three bonds can be described by the Karplus equation (Fig. 1).

Although the ^{1}H–^{1}H coupling information can be extracted, in principle, from the one-dimensional ^{1}H spectrum, this laborious process has been greatly simplified by the introduction of two-dimensional NMR experiments (Jeener, 1971; Aue et al., 1976; Freeman and Morris, 1979) that were later followed by experiments with even higher dimensionality (reviewed in Oschkinat et al., 1994) including pulsed-field gradients (Willker et al., 1993).

In contrast to the all-abundant ^{1}H, ^{13}C occurs only at an abundance of about 1.1% in natural matter. For stochastic

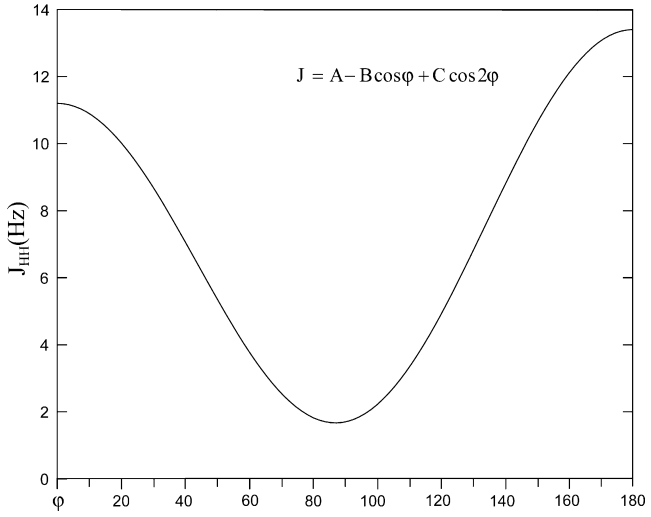

Fig. 1. Graphical display of the Karplus equation (see inset), where J is the ^3J coupling constant, φ is the dihedral angle, and A, B, and C are empirically derived parameters whose values depend on the atoms and substituents involved.

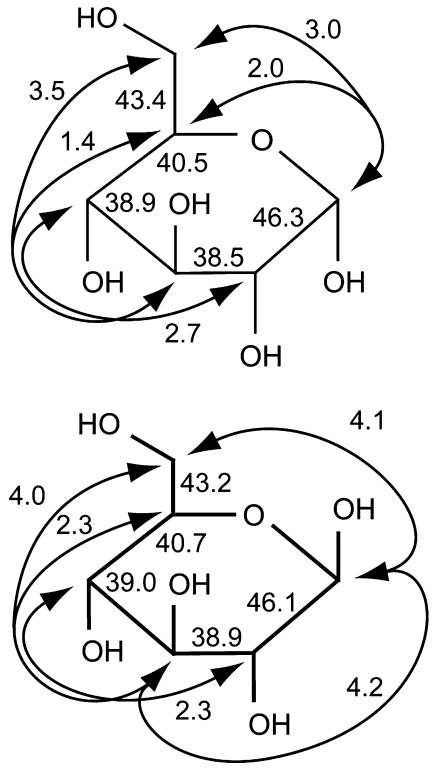

Fig. 2. ^{13}C–^{13}C coupling patterns detected by ^{13}C NMR spectroscopy of α- and β-glucose. The numbers indicate coupling constants in Hz. Couplings via more than one bond are symbolized by double-headed arrows.

reasons, most carbon atoms neighbouring a given ^{13}C atom are ^{12}C. Therefore, ^{13}C–^{13}C coupling information is not easily obtained except in cases where the ^{13}C abundance is artificially enhanced. However, observed ^{13}C nuclei are typically coupled to adjacent ^{1}H atoms, and that information is best extracted by multinuclear multidimensional NMR experiments that have been developed over the past three decades (see below). Carbon coupling constants with values >1 Hz can be detected in practice and this again limits the observable coupling pattern to carbon pairs comprising atoms with a distance of a maximum of three bonds in typical cases. For an example of a ^{13}C–^{13}C coupling pattern which is detectable by high-resolution NMR spectroscopy, see Fig. 2.

The combined application of advanced NMR spectroscopy allows the rapid mapping of spin networks yielding information on the homotopic ^{1}H–^{1}H relations and on the heterotopic ^{1}H–^{13}C relations, whereas the homotopic ^{13}C–^{13}C relations remain typically unknown, due to the low abundance of ^{13}C in natural organic matter. In order to determine the chemical structure of an unknown molecule, the (necessarily incomplete) information on spin topology must be translated into a plausible chemical structure, a process in which a general knowledge of organic chemistry plays an important role. For the structural analysis of progressively larger organic molecules, it is especially important to note that only the nearest neighbour aspects of a given spin network can be assessed from a given "point of view", as defined by whichever nucleus is being observed in an NMR experiment. Since the macrostructure of the spin network cannot be gleaned in its entirety, except in case of the smallest organic molecules with a size range that may extend up to about 100 Da in the most favourable cases, the experimentally observable spin network is best described as a patchwork of microdomains, and the macrostructure must be put together by assembling the pieces of this puzzle: by comparison with X-ray crystallography, NMR can be described as nearsighted (although not, for that matter, shortsighted).

As already mentioned, the most rapid approach for the assessment of the spin network mosaic, as described above, is to employ NMR experiments in more than one dimension. There is now a real armada of different multinuclear and multidimensional experiments that are designed for different applications (for reviews see Kessler et al., 1988; Wrobel et al., 2006; Freeman and Kupce, 2006). In fact, the history of NMR spectroscopy since 1980 has been dominated by the development of ever more sophisticated multidimensional and multinuclear experiments. A Nobel Prize was awarded to Kurt Wüthrich in 2002 for the development and use of NMR technology in the structure determination of biological macromolecules ("for his development of nuclear magnetic resonance spectroscopy for determining the three-dimensional structure of biological macromolecules in solution").

Whereas a one-dimensional NMR experiment involves a single broadband radiofrequency pulse followed by an observation period for the recording of the ensuing free induction decay, a minimum of two consecutive broadband pulses precede the recording of the free induction decay in experiments with more than one dimension. The respective pulses can be separated by intervals of variable length, and it is the length of these respective intervals that provides each additional time domain (one domain per variable delay), in addition to the time domain for the recording of the FID. The processing of the raw data consists in the sequential Fourier transformation of the experimental data set in each respective time domain. The output appears typically as a contour plot which describes signal intensity as a function of two or more chemical shift variables (see Fig. 3 for examples).

It is worth noting that the chemical shift range of ^{1}H, which typically constitutes at least one of these independent variables, is small and hardly extends beyond 10 ppm. Hence, the ^{1}H dimension of multinuclear experiments is crowded. However, the introduction of additional domains is a very powerful tool that allows the spreading of the overlapping signals over one or more additional frequency/chemical shift domains. By comparison, the chemical shift range of ^{13}C is larger by a factor of about 20 (extending over about 200 ppm). For that reason, the ^{13}C chemical shift dimension is particularly powerful for the deconvolution of the crowded ^{1}H signals in multinuclear spectra, by the introduction of a ^{13}C frequency/chemical shift domain in multinuclear experiments. It is also noteworthy that the "range of view" for ^{1}H–^{13}C coupling

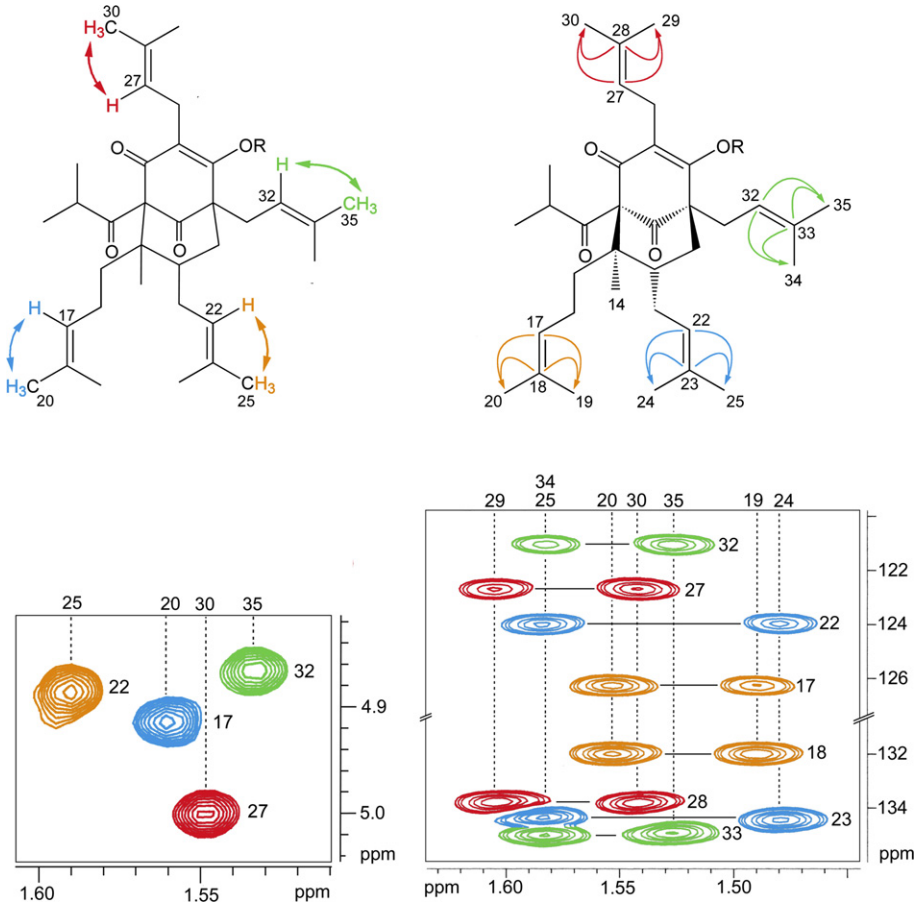

Fig. 3. Expansions of two-dimensional NMR experiments for the metabolite, hyperforin from *Hypericum perforatum*. The left panel displays a part of a NOESY spectrum showing correlations through space between a methyl group and a methine hydrogen atom on the same prenyl moiety in the molecule (see inset). The right panel displays a part of a HMBC spectrum showing correlations between ^{13}C and ^{1}H via long-range couplings through bonds (see inset).

typically extends over either two or three bonds (four bonds are very rare). The chemical shift ranges of ^{31}P and ^{15}N are also relatively large; the latter is particularly important for the NMR analysis of ^{15}N-labelled protein samples.

4. Structure analysis of small natural products

In the first half of the 20th century, the structure elucidation of natural products including vitamins A, B_1, B_2, B_{12}, C, D, and K, porphyrins, bile acids, steroid hormones, alkaloids and terpenes were all outstanding milestones, representing lifetime achievements of research pioneers and their groups that were quickly honoured with the Nobel Prize.[1] With the exception of vitamin B_{12}, those pioneering achievements were obtained by the painstaking chemical disassembly and reassembly of the molecules under study, notably molecules in the molecular weight range below 1000 Da (with the exception of vitamin B_{12} that has a molecular mass of 1355 Da and whose structure was no longer determined by chemical degradation and synthesis of the target compound, but by the emerging technique of X-ray structure analysis). This type of structural organic chemistry based on structure determination by chemical modulation is now extinct and has been replaced by an

[1] Richard Martin Willstätter "for his researches on plant pigments, especially chlorophyll", Chemistry 1915; Heinrich Otto Wieland "for his investigations of the constitution of the bile acids and related substances", Chemistry 1927; Adolf Otto Reinhold Windaus "for the services rendered through his research into the constitution of the sterols and their connection with the vitamins", Chemistry 1928; Christiaan Eijkman "for his discovery of the antineuritic vitamin", Medicine or Physiology 1929; Sir Frederick Hopkins "for his discovery of the growth-stimulating vitamins", Medicine or Physiology 1929; Hans Fischer "for his researches into the constitution of haemin and chlorophyll and especially for his synthesis of haemin", Chemistry 1930; Paul Karrer "for his investigations on carotenoids, flavins and vitamins A and B_2", Chemistry, 1937; Walter Norman Haworth "for his investigations on carbohydrates and vitamin C", Chemistry, 1937; Richard Kuhn "for his work on carotenoids and vitamins", Chemistry 1938; Adolf Friedrich Johann Butenandt "for his work on sex hormones", Chemistry 1939; Leopold Ruzicka "for his work on polymethylenes and higher terpenes"; Chemistry 1943; Edward Adelbert Doisy "for his discovery of the chemical nature of vitamin K", Physiology or Medicine 1943; Sir Robert Robinson "for his investigations on plant products of biological importance, especially the alkaloids", Chemistry 1947; Dorothy Crowfoot Hodgkins "for her determinations by X-ray techniques of the structures of important biochemical substances", Chemistry 1964.

extremely powerful trio of physical methods including multinuclear NMR spectroscopy, mass spectrometry and X-ray crystallography. Alone or in concert, these methods have now the potential to resolve the constitution of the largest existing non-polymeric natural products (i.e. excluding proteins, nucleic acids and complex carbohydrates which are discussed below). Moreover, issues of configuration can be addressed by the joint or single application of X-ray crystallography and NMR, without any limit to the number of stereocenters in the molecule under study. Finally, steady state conformations can be addressed in the solid state by X-ray crystallography and in solution by NMR. Importantly, the time required for structure analysis using these physical methods is a tiny fraction of that required for chemical structure determination. Structural chemistry in the traditional sense has been replaced by molecular physics in a time span of few decades, and the breathtaking development of NMR technology has played a dominant role in this development. It appears safe to say that NMR and X-ray crystallography are now the two most important tools of non-polymeric natural product structure analysis.

Although a very large number of NMR experiments is now available, a small subset is usually sufficient to determine the structures of low molecular weight metabolites (i.e. <1000 Da) (reviewed by Bross-Walch et al., 2005). In typical cases, two-dimensional ^{1}H–^{1}H correlation experiments, such as COSY (Piantini et al., 1982) or TOCSY (Braunschweiler and Ernst, 1983; Bax and Davis, 1985a; Davis and Bax, 1985a,b; Griesinger et al., 1988) recorded with different mixing times, are used to assign the proton spin systems via scalar couplings. Proton-detected ^{1}H–^{13}C correlation experiments with gradient selection of magnetization transfer, e.g. HMQC (Müller, 1979; Bax et al., 1983), HSQC (Bodenhausen and Ruben, 1980) or HMBC (Bax and Summers, 1986) are used to correlate the ^{13}C chemical shifts with protons via one or two/three bonds, respectively. If required, two-dimensional NOESY (Macura and Ernst, 1980) or ROESY (Bothner-By et al., 1984; Bax and Davis, 1985b) experiments provide information about dipolar couplings through space. On the basis of these experiments, a set of constraints for the constitution and the configuration of a molecule can be obtained which may provide sufficient information to unambiguously assign the structure of a typical metabolite. Using advanced NMR equipment, this set NMR experiments can be performed within minutes or hours (depending on the sample concentration). As a consequence, spectrometer time is not the time limiting factor for structure determination of a small natural product.

5. Macromolecular structure

The covalent structures of proteins and nucleic acids can typically be determined by rapid and very elegant biochemical methods. Most notably, DNA sequences can be determined at very high-speed by Sanger post-Sanger technology. Protein sequence is now typically predicted from gene sequences but can also be determined directly by Edman sequencing of mass spectrometry. Whereas sequences can be obtained, in principle, from NMR data and can be read directly from crystallographic electron densities at the highest available resolutions, it is almost universal practice to work with biochemically-determined polymer sequences; the determination of three-dimensional macromolecular structure by X-ray crystallography as well as by NMR spectroscopy is therefore almost entirely focused on conformational analysis.

The 1980s witnessed a controversial discussion as to or not crystal structure analysis can adequately describe the physiological state of proteins in solution. That ghost has been quietly laid to rest in the meantime for a variety of reasons. Although it may happen occasionally that lattice interactions enforce a non-physiological conformation on a crystal, the parallel study of proteins by NMR and X-ray crystallography has afforded similar solutions in a sufficient number of cases to document the general equivalence of the two methods. Moreover, the catalytic activity of many proteins in the crystal state and the successful application of crystal structure analysis for the rational development of drugs leave no doubt about the functional relevance of crystal structures. Last but not least, typical protein crystals contain water at a high-percentage that may even exceed the water concentration of the cytoplasm. The decision to use X-ray or NMR structure analysis for a given problem is therefore pragmatic rather than a question of principal. With that in mind, some general aspects of biopolymer structure analysis are described below.

Conformational NMR analysis is mainly based on the application of the nuclear Overhauser effect (a through-space effect, which should be distinguished from coupling through chemical bonds). Briefly, after radiofrequency excitation the relaxation rate of nuclear spins is modulated by nuclear magnetic dipoles and multipoles in their vicinity. For ^{1}H nuclei, the effective maximum radius for induced relaxation is about 5 Å. Multidimensional experiments are used for the quantitative assessment of NOEs for the largest possible number of ^{1}H nuclei in biopolymers. The interpretation of these data requires the assignment of ^{1}H signals numbering hundreds to thousands. That assignment is now almost invariably based on multinuclear experiments. More specifically, the relatively large chemical shift ranges of ^{15}N and ^{13}C are used for the deconvolution of the highly crowded ^{1}H spectra. In light of the poor sensitivity of ^{13}C and ^{15}N spectroscopy, the efficient application of this approach requires the use of proteins labelled with ^{13}C and/or ^{15}N. The labelling can be uniform, but selective labelling has become progressively more important in recent years (for an example, see Sprangers and Kay, 2007). Both uniform and selective labelling require the recombinant expression of the protein under study in relatively high-yield. Even with these technologies, the comprehensive ^{1}H signal assignment of moderately large proteins is a daunting task.

Once assignment and NOE determination has been completed for a large number of ^{1}H nuclei in the protein under study, the overall structure of the folded protein must be estimated by combining large numbers of short-range distances (maximally up to 5 Å) as gleaned from the relaxation data. The general nearsightedness of NMR observation that has already been mentioned in the study of low molecular weight study compounds becomes progressively more daunting as the size of the molecule becomes larger (similar to the task of investigating the structure of an elephant using a very short ruler).

NMR structure analysis is troubled by a number of additional factors intrinsic to the very nature of the biopolymers under study. For many NMR techniques, the line width and, hence, spectral resolution as well as sensitivity are negatively influenced by the reduction in the rotational velocity of progressively larger molecules in solution. Since sensitivity requirements imply protein solutions at high-concentration, in the range of mM, the concomitant viscosity increase causes an additional reduction in rotational correlation rates. Whereas cytoplasmic proteins are intuitively viewed as hydrophilic species, it turns out that solubility limitations can be a severe handicap for NMR structure analysis.

Despite all these handicaps, a large and complex arsenal of workarounds has been developed in recent years, including the establishment of large NMR facilities, such as the RIKEN Institute at Yokoyama, Japan (Busso et al., 2005; Cyranoski, 2006). Important technical innovations in NMR protein structure determination include: selective stable isotope labelling, a flood of novel pulse sequences including technologies which enable measurements of relatively large proteins (Kay, 2005; Riek, 2003), techniques to partially orient protein molecules (Luy and Kessler, 2006), and solid state NMR technology (Aliev and Law, 2005). However, with one remarkable exception describing some structural features and dynamics of the 670 kDa core particle of the proteosome (Sprangers and Kay, 2007), NMR structure analysis continues to be predominantly limited to the study of relatively small proteins, in the size range up to 100 kDa. Luckily, recombinant protein technology can often be used to carve individual protein domains out of larger proteins that can then be addressed with reasonable ease.

By contrast to NMR, X-ray crystallography has not hit any general limit with respect to size and molecular complexity. The X-ray structure of myoglobin, the first macromolecule ever to have its structure determined, was published by Perutz and Kendrew in 1958 after several decades of pioneering work (honoured by the Nobel Prize in Chemistry in 1962). In more recent times, X-ray crystallography has benefited enormously from the construction of synchrotron beam lines with high luminosity and from numerous other technical advances. Over the years, the number of published biopolymer structures has been growing at a quasi-exponential rate and is now in the range of tens of thousands. Various large structures in the MDa range have been solved crystallographically, including that of the ribosome (Cate et al., 1999; Ban et al., 2000; Selmer et al., 2006; Korostelev et al., 2006) and the proteosome (Groll et al., 2000). At the present time, the Brookhaven Protein Data bank (http://www.rcsb.org/pdb/home/home.do) presents 34,259 protein structures solved by X-ray analysis, 5375 protein structures solved by NMR analysis, and 101 protein structures solved by electron microscopy (as of May 08, 2007).

Plant protein structures constitute only about 4% (in total about 1500) of the entries in the Brookhaven Protein Data Bank indicating a rate which is still marginal for plant proteins in crystallography and structural biology. The fraction of plant protein structures that have been determined by NMR is in the range of 10% of all plant protein structures. In many cases, the plant protein NMR structures were obtained from recombinant domains of larger proteins in order to reduce size.

6. Metabolomics

The term metabolomics refers to the systematic study of the unique chemical fingerprints that specific cellular processes leave behind and specifically, the study of their small-molecule metabolite profiles. The subject has been covered in numerous recent reviews (Last et al., 2007; Verpoorte et al., 2007; Ward et al., 2007; Lindon et al., 2007; Aranibar et al., 2006; Dixon et al., 2006; Schauer and Fernie, 2006; Moing et al., 2004; Fernie, 2003).

In principle, any method for the quantitative analysis of low molecular weight biomolecules, including, but not limited to, liquid and gas chromatography in their many variations, capillary electrophoresis, mass spectrometry, with or without prior derivatization and/or in conjunction with preseparation by liquid or gas chromatography, and, last but not least, NMR spectroscopy, can be put to the task.

By comparison with HPLC, GC, CE, MS, GC–MS and LC–MS, the amount of sample required is much larger in NMR spectrometry, as discussed earlier in this article. In the domain of applied plant sciences, however, this is typically not a major concern.

It is immediately obvious from the foregoing discussions that NMR is a powerful technique for the structural identification of metabolites that have been isolated in pure or at least partially purified form using appropriate techniques. With the introduction of flow-through probe heads, it has even become possible to obtain ''in-flight'' NMR spectra of HPLC fractions from biological samples.

It is also possible to record NMR spectra from crude metabolite mixtures and to extract the relative amounts of different metabolites by systematic signal deconvolution. In this context, it is relevant that the chemical shift range for ^{1}H is small, and the ^{1}H spectra of complex mixtures are necessarily crowded. By comparison with ^{1}H, the chemical shift range for ^{13}C is about 20 times larger. Moreover, ^{13}C signals of compounds with natural ^{13}C abundance

appear as singlets after proton decoupling, as opposed to the multiplet structure of most ^{1}H signals. For both reasons, the ^{13}C domain of metabolite mixtures is less subject to overcrowding by comparison with the ^{1}H domain.

As described in the section on protein structure determination, NMR offers a powerful means for the deconvolution of crowded spectra by the transition to experiments with two or even more NMR dimensions. It is also possible to link signals of a given compound in a complex mixture by correlation techniques such as ^{1}H–^{1}H correlation and ^{1}H–^{13}C correlation. Each of these techniques affords cross-correlated signals that interrelate specific spectral features that pertain to one given molecule in a mixture. However, the instrument time required becomes substantially larger with each added NMR dimension. Moreover, by comparison with one-dimensional spectra, the accuracy of signal amplitude determination becomes worse.

Last but not least, the chemical shift values of organic molecules in solution are unfortunately not invariable. Rather, they are influenced in subtle ways by a variety of parameters including, but not limited to, temperature, changes (even minor changes) in solvent makeup, and the concentration of the compound under study as well as all other compounds present in the mixture. This subtle variability can be a major hurdle for computerized spectra evaluation (Daviss, 2005).

With all that said, metabolomics by NMR is not, *per se*, a novel or specific scientific domain. It is simply the application of the ''tricks of the trade'' of NMR analysis to more or less complex biomolecular mixtures. As a general caveat, reproducibility and accuracy of quantitative NMR determinations come at the price of a relatively high-investment in instrumentation, time and effort. By comparison, rigorous standardization is more easily achieved, for example, with HPLC methods.

On the positive side, however, NMR has numerous advantages by comparison with other methods that should be viewed as complementary rather than competitive. In contrast to GC–MS, extracts can be directly analyzed by NMR analysis without prior derivatization and metabolite concentrations can be determined in an unbiased manner.

Even if all these concerns can be appropriately addressed, the problem remains that high-volume analytical data are not *per se* the answer to most practical problems in applied plant science. The questions is whether it is possible to ''make sense'' of the large data volumes that can be generated by the powerful analytical armamentarium at our fingertips. Data can always be correlated in one way or another, but it is a noteworthy triviality that correlation should not be misinterpreted as causality.

7. Biosynthetic pathways

A major surprise from whole genome sequencing was the discovery that higher plants have larger numbers of genes as compared to vertebrates (*Arabidopsis thaliana*, 28,000; rice, 50,000; poplar, 40,000; vertebrates, 21,000–24,000). With hindsight, this can be seen in part to be due to the complexity of plant metabolism and the consecutive requirement for large numbers of enzyme catalysts. This is squarely at odds with the fact that at least the primary metabolism of plants was in the shadow of mammalian and bacterial metabolism for decades, since textbooks had a tendency to convey the idea that vertebrates and bacteria were the universal model organisms. It is now becoming progressively clear that this is a baseless oversimplification.

On the other hand, it has always been duly appreciated that plants are an abundant treasure trove of secondary metabolites, in many cases with complex structures. The interest in plant secondary metabolites was in part driven by medical aspects. In fact, plant preparations are the oldest effective drugs in human history, and pure preparations of plant metabolites continue to provide important drugs such as atropine, taxol and artemisinin.

Knowledge about biosynthetic pathways is the prerequisite for the production of natural compounds by optimized bio-technological means. Tracer studies using isotopes are among the most important methods for the study of biosynthetic pathways in both primary and secondary metabolism. For decades, the area was dominated by radioactive tracers which could be detected with high-sensitivity and accuracy using comparatively simple techniques. Although stable isotopes are, in principle, easier to work with, due to the absence of safety restrictions, their application for biosynthetic work had to await sensitive and specific detection methods, i.e. advanced mass spectrometry and high-resolution NMR spectrometry.

Biosynthetic isotope tracer studies in plants have a rich history of successes that is checkered with some remarkable glitches. Thus, experiments with $^{14}CO_2$ were crucial for the Nobel Prize winning work on photosynthesis by Calvin and his group in 1961 (for a review, see Bassham, 2003). On the other hand, isotope studies were misinterpreted over several decades as evidence of the universality of the mevalonate pathway for the biosynthesis of plant isoprenoids, when in reality most of these arise predominantly from building blocks derived from the non-mevalonate pathway, whose existence was stubbornly ignored in the face of clear-cut experimental evidence to the contrary (reviewed in Eisenreich et al., 1998). This section is focussed on the question of how such errors came about, and how they can be reliably avoided in future.

Isotope incorporation studies, irrespective of the radioactive or non-radioactive character of the tracer, are most of the time interpreted in terms of simple source-and-sink models (Fig. 4). If the isotope from the proffered tracer (i.e. the source) is diverted to the compound under study (i.e. the sink), it is logical to conclude that there must be some metabolic pathway that explains this result. This obvious truth is frequently overinterpreted with the intention of proving that a given pathway provides the

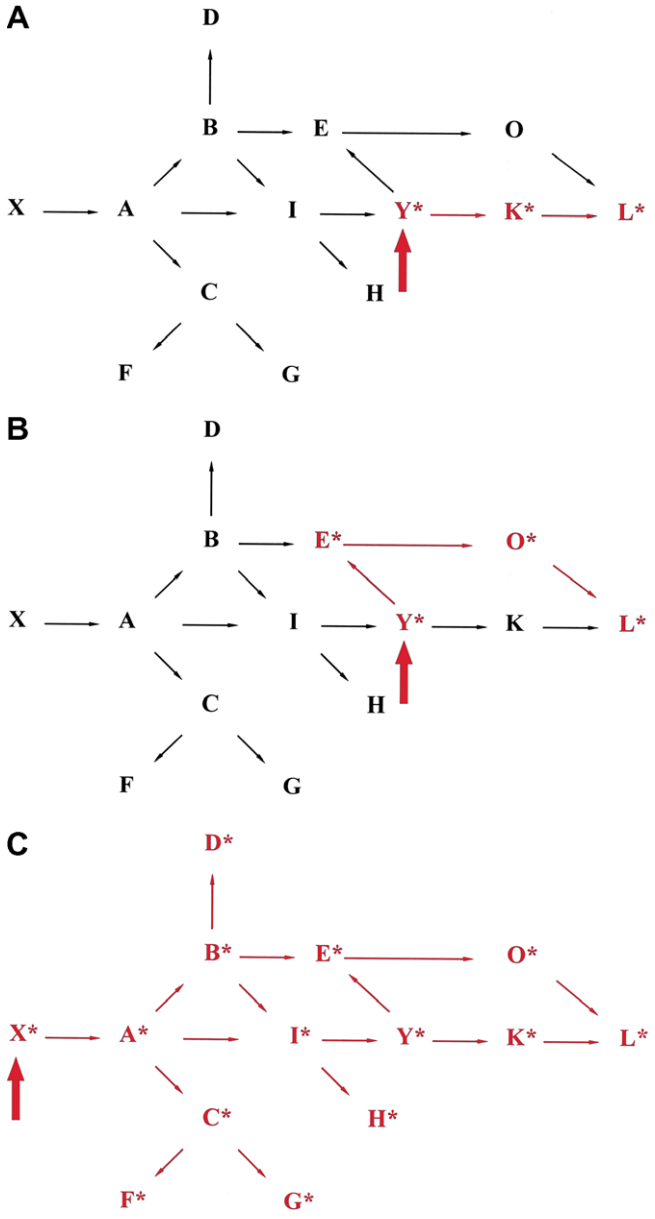

Fig. 4. Section of a metabolic network. A and B: In a source-and-sink model, an isotopically-labelled compound Y is applied to an organism. The fact that the target metabolite L acquires label is typically explained by a close biogenetic relationship of Y and L (case A), but can also be a consequence of a more complex biosynthetic history of L where Y is converted into intermediate E that serves as a precursor of L via a completely different pathway (case B). With a general precursor (such as glucose or CO_2) most if not all metabolites in the network acquire label (case C). The complex but specific isotopologue profiles can be deconvoluted by advanced bioanalytical methods (i.e. quantitative NMR spectroscopy and mass spectrometry). With multiple isotopologue profiles at hand, biosynthetic pathways and metabolite flux can be assessed on a quantitative basis.

dominant or only contribution to the formation of a product. In fact, the diversion of an isotope from a given source to a given sink can vary over many orders of magnitude. If the transfer rate is high or very high, there can be hardly any doubt about the existence of a close metabolic connection. On the other hand, if the transmission is in the low percent range or even lower, the result is open to many different interpretations.

The fallacy of oversimplified source-and-sink models should be obvious even after a brief inspection of the standard diagrams of intermediary metabolism: Intermediary metabolism is a complex network and not an array of parallel highways. In a more rigorous description, cellular metabolism is a scale-free network, where a given node can be reached from virtually any other node via a relatively small number of intermediary nodes. Notably, however, there is frequently more than one possible connection from any source node to any sink node (cf. Fig. 4A and B). The problem in biosynthesis research is not to find any road from one metabolite to a second metabolite but to find the main road where most of the traffic takes place, and possibly additional roads with less traffic. This will become more obvious from the example that is reported in some detail below.

The following is a brief introduction to the concept of isotopologue space as a useful tool for the interpretation of biosynthetic tracer experiments. As described above, natural organic matter comprises the two stable isotopes of carbon, ^{12}C and ^{13}C, in an approximate ratio of 99:1. The two isotopes are normally distributed to different molecular positions in an almost random (although not strictly random) fashion. As a consequence, any pure organic compound is, on closer analysis, a complex mixture of different isotopologues.[2] Similar considerations apply to the stable isotopes of hydrogen and nitrogen, but the present discussion can be limited to carbon isotopes.

For any organic molecule comprising n carbon atoms, the number of carbon isotopologues is 2^n, and the isotopologue space for that molecular species has 2^n dimensions. For example, natural glucose consists of 64 carbon isotopologues, where [U-$^{12}C_6$]-glucose is the most abundant species that accounts for about 93 mol% and each molecular species carrying a single ^{13}C atom accounts for about 1.1 mol%. Molecular species carrying more than one ^{13}C atom are progressively rare in glucose with natural ^{13}C abundance; specifically, the [U-$^{13}C_6$]-isotopologue has an abundance of about 10^{-10} mol% (Fig. 5). The abundance of isotopologues can be interpreted as a concentration that is best expressed in the mol% dimension.

The natural, quasi-random distribution of different isotopes to different molecular positions can be modulated by the introduction of a specific isotopologue, a mixture of isotopologues, or a mixture of compounds comprising single or multiple isotopologues (for the example of glucose that has been introduced above, the abundance of the [U-$^{13}C_6$]-isotopologue can be increased experimentally by more than 10 orders of magnitude).

[2] Isotopologues are molecular species of a given chemical compound that differ in isotope composition (number of isotopic substitutions), whereas isotopomers differ in the position of isotopic substitution(s) but not in the net isotope composition. In that sense, sets of isotopomers are subsets of the isotopologue superset; isotopomers are also designated as positional isotopologues by certain authors.

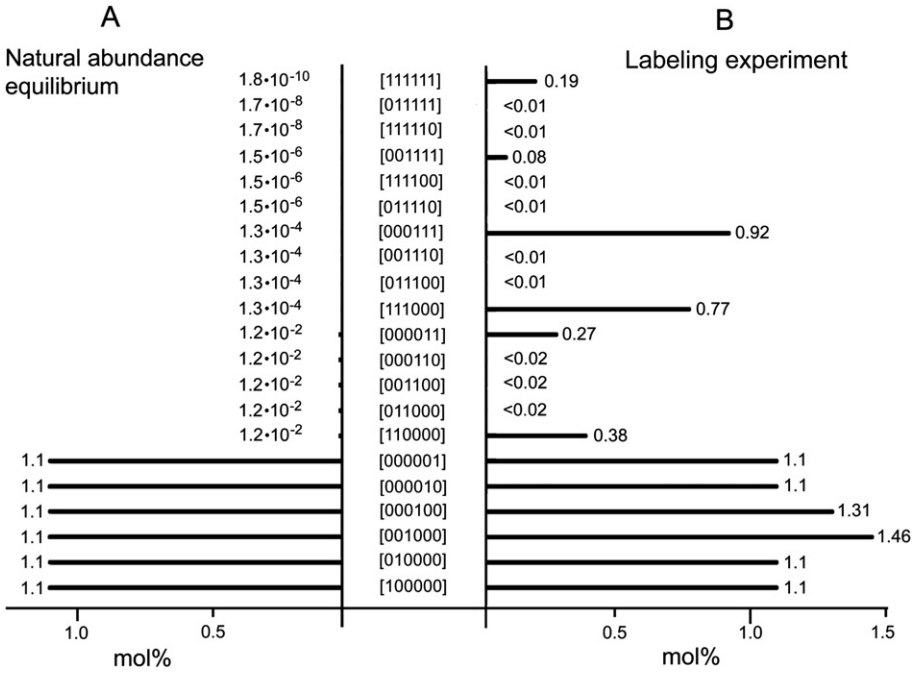

Fig. 5. Molar abundance of ^{13}C isotopologues of glucose (1 = ^{13}C, 0 = ^{12}C; the first digit indicates C-1, the second digit indicates C-2, etc.). (A) In natural abundance material and (B) from starch hydrolysates of maize kernels supplied with [U-$^{13}C_6$]-glucose and unlabelled glucose at a ratio of 1:40 (w/w) (Glawischnig et al., 2002).

In the wake of an experimental perturbation of the natural quasi-equilibrium distribution of isotopologues, a relaxation process takes place that is progressively conducive to the re-establishment of the stochastic isotope distribution. In principle, all metabolic processes in a given metabolic network can contribute to that relaxation process that can be followed in time by the analysis of the isotopologue composition of one or, preferably, multiple metabolites. Using present-day instrumentation, this daunting task is best addressed by NMR spectroscopy, alone or in conjunction with mass spectrometry.

Ideally, a comprehensive isotopologue perturbation/relaxation analysis would involve the assessment of all isotopologues of all low molecular weight metabolites in a given experimental system. This is as yet not possible with an acceptable experimental effort. However, numerous examples have documented that the quantitative assessment of the most abundant isotopologues of a relatively small number of metabolites can be sufficient for the elucidation of complex metabolic pathways. Notably, the building blocks for complex natural products are extracted from a relatively small set of central metabolic intermediates and primary metabolites, and these are metabolically connected by known pathways of intermediary metabolites (Fig. 6).

Since the number of isotopologues is large, except for the smallest metabolites, it appears appropriate to use a simple nomenclature system for their description. We prefer the designation of isotopologues by binary numbers where each digit represents a certain carbon atom, and ^{12}C and ^{13}C are designated by 0 (zero) and 1 (one), respectively. As a specific example, $\{110000\}_{glucose}$ designates a glucose isotopologue carrying ^{13}C in positions 1 and 2, whereas the other positions are occupied by ^{12}C. Sets of isotopologues where the occupancy at certain positions is unknown can be expressed in this notation using a wildcard X representing either ^{12}C or ^{13}C. For example, $\{110XXX\}_{glucose}$ represents the set of eight glucose isoto-

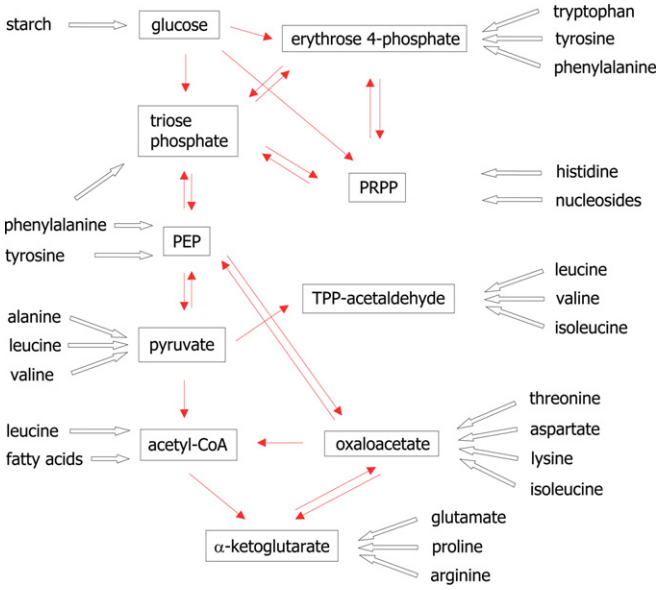

Fig. 6. Reconstruction of isotopologue compositions in central intermediates (compounds indicated in boxes) by retrobiosynthetic analysis of sink metabolites (e.g. amino acids) in plants. PRPP, phosphoribosyl pyrophosphate; TPP, thiamine pyrophosphate; PEP, phosphoenol pyruvate.

pologues where positions 1 and 2 are occupied by ^{13}C, position 3 is occupied by ^{12}C, and the occupancy of the other carbon atoms is undetermined. Interestingly, the sum of certain X-groups of this type is directly proportional to the integrals of certain satellite signals in ^{13}C–^{13}C-coupled ^{13}C NMR spectra. These integrals therefore represent linear combinations of isotopologue abundances, and the abundances of individual isotopologues can be extracted from X-groups by linear deconvolution. In the case of glucose, a set of approximately 40 X-groups can be quantified by NMR (Fig. 7) (Eisenreich et al., 2004a).

A recent expansion of the X-group concept that facilitates the combination of data from NMR and mass spectrometry as the basis for isotopologue abundance analysis by linear deconvolution concerns the introduction of a second wild card denominator Y (Römisch-Margl et al., 2007). Specifically, Y can be either ^{12}C or ^{13}C, but the sum of all Y denominators in a given isotopologue set has a specific value. In the glucose example, $\{110YYY\}^{y=1}$ designates isotopologues that carry a total of three ^{13}C atoms, two ^{13}C atoms at positions 1 and 2, one ^{13}C at either of one of the positions 4, 5 or 6, and ^{12}C at position 3.

These concepts allow complete isotopologue dissections for metabolites comprising up to four carbon atoms and partial dissections for larger molecules (Römisch-Margl et al., 2007). Since large metabolites invariably arise as mosaics by the assembly from smaller modules (for example, terpenes invariably arise by assembly from the common five carbon precursors, isopentenyl diphosphate and dimethylallyl diphosphate), even partial isotopologue dissection can go a long way in biosynthetic pathway analysis.

Since the overall amounts of certain central metabolic intermediates such as carbohydrate phosphates are relatively low, it is often convenient to reconstruct their labelling patterns from abundant primary metabolites such as glucose obtained from the hydrolysis of starch and of amino acids obtained from the hydrolysis of proteins which act as sinks for the overall carbon flux and can be interpreted as a ''fossil memory'' of intermediary metabolism (Szyperski, 1995; Bacher et al., 1999). For example, since the shikimate pathway is the unique route for the biosynthesis of proteinogenic aromatic amino acids, the labelling pattern of phosphoenol pyruvate can be extracted from the side chains of tyrosine and phenylalanine, and the labelling pattern of the tetrose phosphate intermediate can be extracted from the labelling patterns of the aromatic rings. Similarly, the isotopologue abundances of pyruvate, oxaloacetate and 2-ketoglutarate can be gleaned from the isotopologue abundances of alanine, aspartate and glutamate, respectively. On the basis of known pathways of amino acid biosynthesis, the labelling pattern of central intermediates can be reconstructed from additional amino acids, as shown in Fig. 6.

Using these data, labelling patterns can easily be predicted in order to test hypotheses for the biosynthesis of natural products from the chemical inventory of plant metabolites. This retrobiosynthetic approach to the study of biosynthesis has been used successfully for the rapid elucidation of biosynthetic pathways in plants as well as microorganisms. The number of studies employing NMR-based retrobiosynthesis is very large and a comprehensive treatment is far in excess of the scope of the present review. The few examples given below reflect specific areas reflecting the expertise of the authors.

An early example of a retrobiosynthetic analysis of plant metabolites was the delineation of gallic acid biosynthesis. The biosynthesis of gallic acid (**4**, Fig. 8) has been controversial for several decades. Although there was general agreement that it involved the shikimate pathway, the committed precursor was variously assumed to be either phenylalanine (**1**) (Zenk, 1964; Dewick and Haslam, 1968) or an earlier intermediate or product of the shikimate pathway, such as dehydroshikimate (**2**) (Dewick and Haslam, 1968, 1969) or protocatechuate (**3**) (Kato et al., 1968; Chandran and Frost, 2001), respectively (Fig. 8). This controversy was resolved by *in vivo* studies with the fungus, *Phycomyces blakesleeanus* and the plant, *Rhus typhina* (Werner et al., 1997). Experiments were performed where either the fungus was grown on a medium containing a mixture of [U-$^{13}C_6$]-glucose and a large excess of glucose at natural abundance or where cut plant segments were immersed into a solution containing the same glucose mixture. The culture or the plant segments were held for several days under these conditions and gallic acid, as well as protein-derived phenylalanine was isolated and analyzed by NMR spectroscopy. On the basis of the isotopologue compositions of phenylalanine (**1**), the patterns in the basic shikimate building blocks, erythrose 4-phosphate (**5**) and phosphoenolpyruvate (**6**) were reconstructed (Fig. 9). In the next step, their labelling patterns were used to simulate the labelling patterns of gallic acid (**4**) via different hypothetical biosynthetic pathways involving either an early shikimate intermediate or the amino acid, phenylalanine, as the potential precursors. The comparison of the observed isotopologue profiles in gallic acid clearly showed that in the fungus as well as in the plant, this compound is derived from the early part of the shikimate pathway and that phenylalanine is not the committed precursor (Fig. 9).

More recently, the biosynthetic pathway of gallic acid in leaves of *R. typhina* has been studied by ^{18}O isotope ratio mass spectrometry at natural oxygen isotope abundance (Werner et al., 2004). The observed $\delta^{18}O$-values of gallic acid implied biogenetical equivalence with oxygen atoms of carbohydrates but not with oxygen atoms introduced by monooxygenase activation of molecular oxygen. It was concluded that all phenolic oxygen atoms of gallic acid are retained from the carbohydrate-derived precursor 5-dehydroshikimate. This provided strong evidence that gallic acid is indeed synthesized entirely or predominantly by dehydrogenation of 5-dehydroshikimate. In line with this result, it was shown that extracts of birch leaves catalyze the formation of gallic acid from dehydroshikimate at low rates (Ossipov et al., 2003).

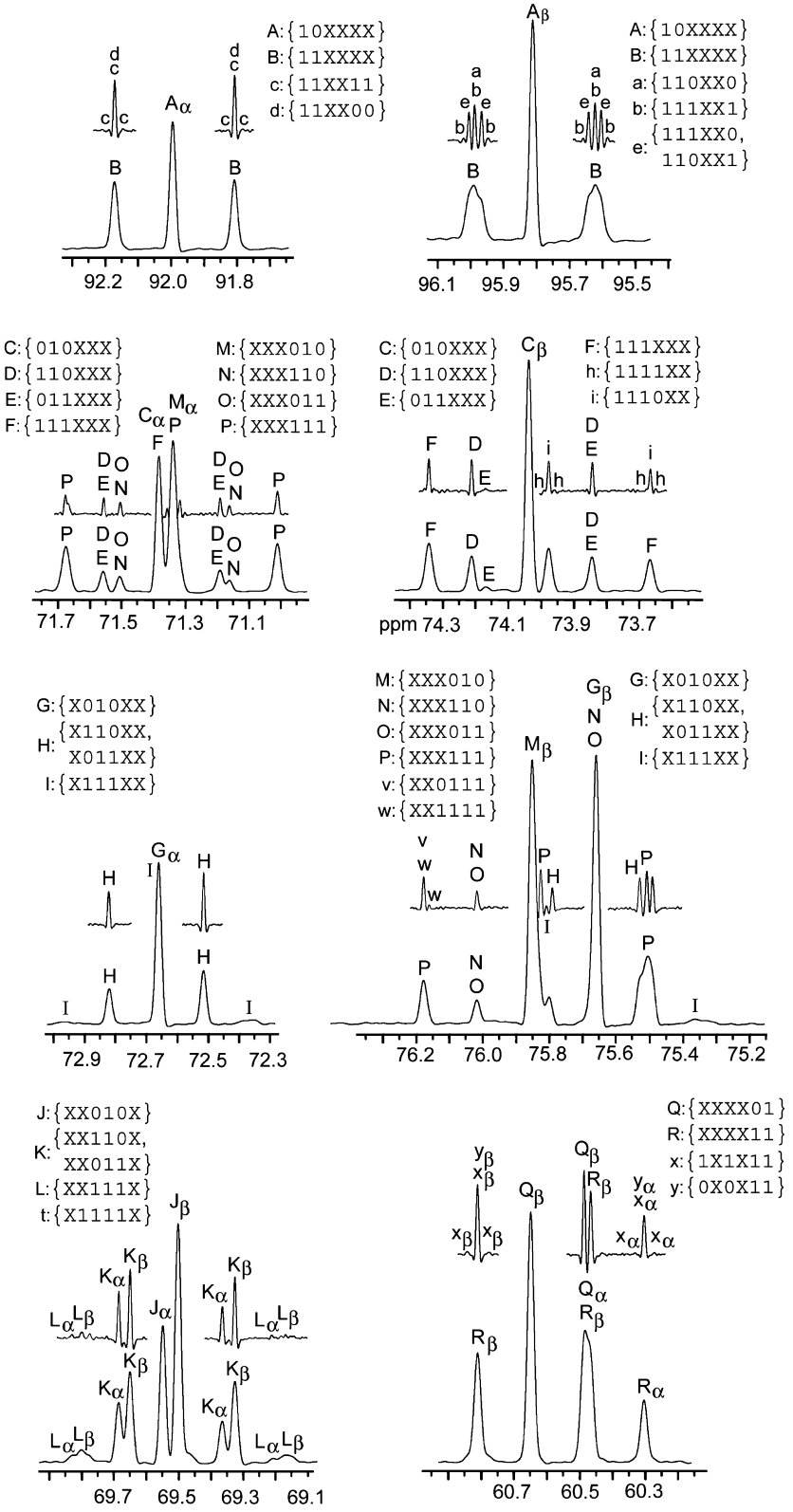

Fig. 7. ^{13}C NMR signals of starch glucose isolated from maize kernels cultivated on a medium containing [U-$^{13}C_6$]-glucose (Ettenhuber et al., 2005b). The signals are assigned to corresponding X groups (1 = ^{13}C, 0 = ^{12}C and X = ^{13}C or ^{12}C; the first digit indicates C-1, the second digit indicates C-2, etc.). For a detailed definition of X groups (see Eisenreich et al., 2004a).

A classical milestone in plant sciences was the elucidation of nicotine biosynthesis in *Nicotiana tabacum*. Source-and-sink experiments clearly indicated that nicotinic acid is a building block in the biosynthesis of the alkaloid (Dawson et al., 1960). More recently, three different pathways for the biosynthesis of nicotinic acid have

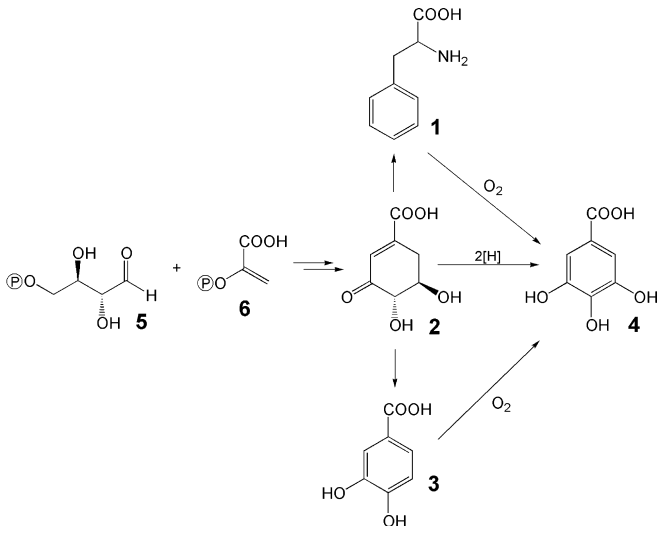

Fig. 8. Possible biosynthetic pathways for the formation of gallic acid (**4**).

been described in nature: (i) starting from dihydroxyacetone phosphate and aspartate as building blocks, (ii) starting from *N*-formyl aspartate and acetyl-CoA, and (iii) starting from tryptophan. It was therefore decided to quantitatively assess possible contributions of the respective pathways in the plant under physiological conditions. A labelling experiment with $^{13}CO_2$ as precursor showed the presence of multiple ^{13}C-labelled isotopologues in nicotine (Römisch-Margl et al., 2007). Retrobiosynthetic analysis of this labelling pattern was perfectly in line with a prediction by nicotinic acid formation via the aspartate pathway (Fig. 10).

Retrobiosynthetic analysis has also been essential to assign the biosynthetic origin of many plant terpenes and isoprenoids. This class of compounds represents one of the largest groups of natural products, including essential

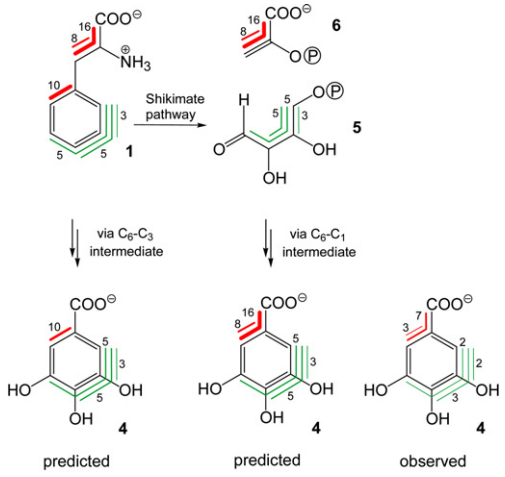

Fig. 9. Observed and predicted labeling patterns of gallic acid from *Rhus typhina* supplied with [U-$^{13}C_6$]-glucose (Werner et al., 1997). Multiple ^{13}C-labelled isotopologues are indicated by bars connecting ^{13}C-atoms within the same molecule. The widths and the numbers indicate the relative abundances of the isotopologues.

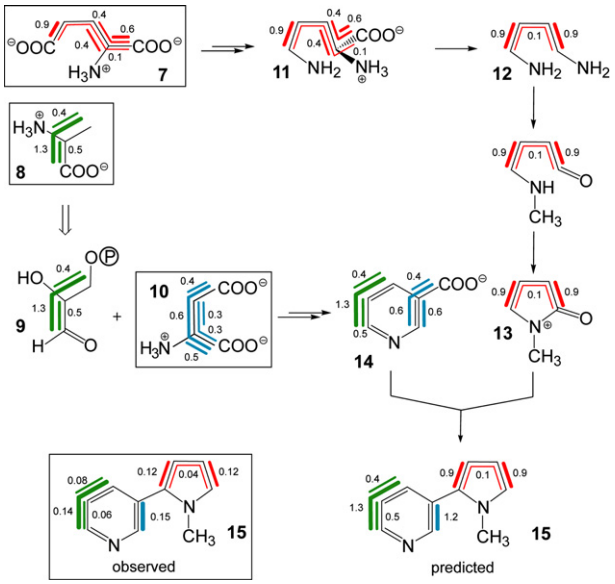

Fig. 10. Isotopologue profiling of nicotine biosynthesis in *N. tabacum* supplied with $^{13}CO_2$ (Römisch-Margl et al., 2007). The isotopologue patterns of alanine (**8**), aspartate (**10**) and glutamate (**7**) were determined by metabolic simulation on the basis of the X-group abundances of glucose and the amino acids. The observed labelling pattern of nicotine (**15**) is inferred from the isotopologue set observed by NMR spectroscopy. On the basis of the labelling patterns in the amino acids, the isotopologue pattern of nicotine is also predicted following known routes of nicotine biosynthesis in *Nicotiana*. Multiple ^{13}C-labelled isotopologues are indicated by bars connecting ^{13}C-atoms within the same molecule. The numbers indicate the abundances of the corresponding isotopologues in mol%. The widths of the bars reflect the relative abundances of the isotopologues. The widths of the bars in nicotine (inferred from NMR spectroscopy) were increased by an approximate value of 10.

plant metabolites such as sterols, carotenoids or the phytol moiety of chlorophylls. In the early 1990s, Arigoni and Schwarz showed that the isotopologue pattern obtained in ginkgolides from feeding [U-$^{13}C_6$]-glucose to seedlings of the tree, *Gingko biloba* was not in line with the mevalonate hypothesis (Schwarz, 1994). This seminal study triggered one of the most exciting developments in plant metabolism during the last decade and led to the discovery of a mevalonate-independent biosynthetic pathway to the universal terpene precursors, isopentenyl diphosphate (IPP, **21**, Fig. 11) and dimethylallyl diphosphate (DMAPP, **20**) via 1-deoxyxylulose 5-phosphate (for reviews, see Rohmer, 2007; Rohdich et al., 2005, 2003).

Follow-up studies using the retrobiosynthetic approach showed that the isoprenoid precursors for the vast majority of plant terpenes and isoprenoids are at least partially contributed from the non-mevalonate pathway (see Eisenreich et al., 2004b and references quoted therein). Whereas the classical pathway proceeds via acetyl-CoA (**22**) and mevalonate (**23**) in the plant cell cytoplasm, the alternative pathway via 1-deoxyxylulose 5-phosphate (**18**) and 2-C-methylerythritol 4-phosphate (**19**) is operative in plastids (Fig. 11). However, there is evidence that at least one as yet unidentified downstream metabolite of the isoprenoid pathway can be exchanged across the compartmental

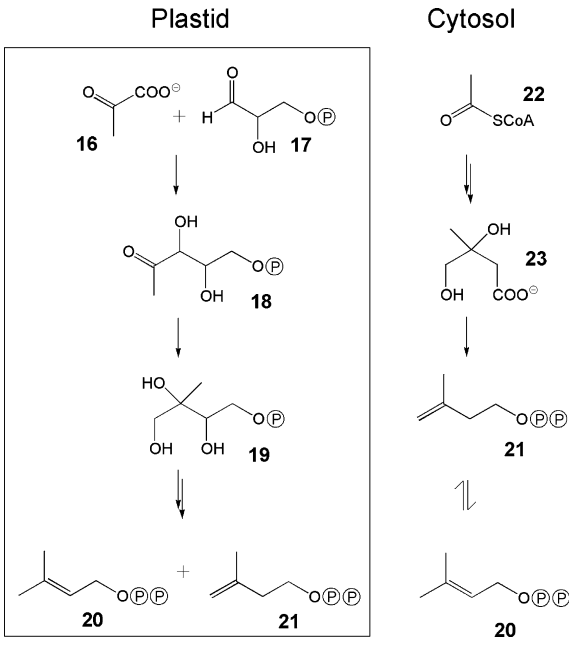

Fig. 11. Topology of IPP (**21**) and DMAPP (**20**) biosynthesis in plants. For more details, see text.

boundary; this frequently results in observed crosstalk between the pathways. Moreover, certain terpenoids appear as mosaics where certain building blocks are predominantly supplied by the mevalonate pathway, whereas others are predominantly supplied by the non-mevalonate pathway (reviewed in Eisenreich et al., 2004b).

This introduction to the network concept of metabolism is not intended to detract from the fact that numerous studies have been conducted successfully with the more classical source-and-sink concept. In this regard, it is interesting to address the relative merits and the requirements of the two approaches.

The source-and-sink concept is best suited when a structurally complex precursor can be obtained in appropriately labelled form and can be administered in a way that is conducive to efficient incorporation. Typically, a capability for performing advanced organic synthesis is required in order to obtain the required, isotope-labelled tracer. Moreover, it is essential to correctly select, on the basis of educated guessing, a tracer compound that is either a pathway intermediate or can be converted into a pathway intermediate by enzyme reactions in the experimental system under study. The NMR requirements can be relatively modest. In typical cases, it is sufficient to show that one signal of the sink compound is enhanced by comparison with its natural abundance intensity, or that a group of connected ^{13}C atoms is jointly incorporated into the target compound, as evidenced by ^{13}C–^{13}C coupling.

On the other hand, simple and easily accessible tracer compounds are typically sufficient for the retrobiosynthetic approach. The central concept of retrobiosynthetic analysis consists of the generation of a deviation from the quasi-random isotopologue distribution and the analysis, as a function of time, of the isotopologue distribution as it unfolds in the relaxation process driven by metabolic reactions. For the ultimate analysis, it is not essential how the initial isotopologue disequilibrium is generated. The tracer can be selected in order to optimize utilization by the plant or the plant tissue under study. Remarkably, even $^{13}CO_2$ can be used for this purpose (Schaefer et al., 1975, 1980; Hutchinson et al., 1976; Römisch-Margl et al., 2007). More specifically, generally labelled photosynthate can be generated by a pulse of $^{13}CO_2$, and the consecutive evolution of multiply labelled isotopologues in different metabolic compartments can be followed experimentally in order to elucidate biosynthetic pathways. The technique enables biosynthetic studies under strictly physiological conditions and in situations where more specific precursors cannot be applied, e.g. because appropriate cell cultures are not available and segments of plants do not utilize exogenous tracers with sufficient efficacy. In summary, the retrobiosynthetic approach requires the isolation of multiple metabolites whose NMR spectra must be interpreted and analyzed, and, as a consequence, the NMR and computational requirements for the quantitative multi-isotopologue analysis are high.

Last but not least, the two approaches can be combined, with the retrobiosynthetic methodology serving the rapid identification of the building blocks derived from central intermediary metabolism and the source-and-sink concept being employed for the detailed elucidation of specific and structurally complex pathway intermediates.

8. Metabolite flux

The perturbation/relaxation concept introduced in the previous chapter can be adapted to the study of metabolic flux in plant cell, plant tissues and whole plants. As already stated, the introduction of an isotope-labelled metabolite or a mixture of labelled metabolites into a given experimental system generates a local perturbation of the isotopologue distribution in the network which will spread through the entire network by relaxation processes, driven by a wide variety of enzyme reactions. The isotopologue composition of various metabolites can then be assessed as a function of time and provides information on specific transformation rates.

Until recently, metabolic flux analysis was predominantly applied to microbial fermentations, frequently with the aim to improve the yield of biomass or of specific metabolites (Sauer, 2005). Data interpretation in case of microbial fermentations is greatly facilitated by the fact that an isotropic distribution of metabolites can be enforced by stirring of the bulk solution and by the possibility of conducting experiments under quasi-steady state conditions in continuous-flow fermentors. Under these conditions, it is in principle possible to control the entire isotopologue balance, and this is advantageous for computational simulation.

More recently, stable isotope experiments monitored by NMR and/or mass spectrometry have been adapted to studies with plant systems. As for studies with microbial fermentations, it is a major aim of these studies to provide a rational basis for the improvement of biomass and/or specific plant products. Although the technique is relatively new in plant sciences, several published have been reported in recent years (Roscher et al., 2000; Ratcliffe and Shachar-Hill, 2001, 2006; Shachar-Hill, 2002; Kruger et al., 2003; Schwender et al., 2004; Krishnan et al., 2005).

Studies with cultured plant cells, plant tissue, such as root or kernel cultures, or plant embryos are technically similar to the study of microbial fermentations. The cells, tissues or embryos can be immersed or submersed in a nutrient medium, and a variety of isotope-labelled precursors can be used to generate a skewed isotopologue distribution that can subsequently evolve by metabolic processes that are interpreted as a relaxation process following the initial perturbation. Specifically or uniformly ^{13}C-labelled glucose and sucrose have been used, but in principle any labelled metabolite that is absorbed by the cells or tissues at a sufficient rate is suitable. As examples, metabolic flux patterns have been reported for tomato cell cultures (Rontein et al., 2002), developing embryos of *Brassica napus* (Sriram et al., 2004; Schwender et al., 2006) and *N. tabacum* (Ettenhuber et al., 2005a), and kernel cultures of *Zea mays* (Glawischnig et al., 2002; Ettenhuber et al., 2005b; Spielbauer et al., 2006).

The application of labelled precursors presents technical problems for studies with whole plants. Injection or infiltration of metabolites such as glucose is possible with tall species such as maize, but the dynamics of the distribution of the tracer in the whole plant is hard to control. On the other hand, photosynthetic labelling with $^{13}CO_2$ provides a means to distribute the label to all photosynthetic parts of an intact plant, irrespective of its size (Schaefer et al., 1975, 1980; Hutchinson et al., 1976; Römisch-Margl et al., 2007). With suitable equipment for the application of $^{13}CO_2$ under physiological conditions, the technique can be used for small species such as *A. thaliana*, as well as for tall species including adult plants of major crops such as corn (*Z. mays*), wheat (*Triticum* spp.), and rapeseed (*B. napus*). As described in the previous section, a variety of metabolites including, but not limited to, glucose (either free or from hydrolysis of starch or cellulose) and amino acids (either free or from hydrolysis of protein) can be isolated and their isotopologue abundance can be determined by NMR and/or mass spectrometry.

Computational modelling of metabolic studies in whole plants is complicated by aspects of topological complexity at the levels of cells, tissues and whole plants. Whereas an analytical *ab initio* solution may be difficult or impossible to achieve with presently available software, the technology appears ripe for comparative studies in which plants with different genetic makeups and/or different experimental conditions are compared.

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Adelbert Bacher served as associate professor of Microbiology at the University of Frankfurt and as professor of Organic Chemistry and Biochemistry at the Technical University of Munich.