NMR of Nature's Plastics and Spiders' Webs: Chemistry, Physics, or Biology?†

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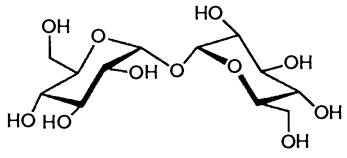
1 Introduction

Science is often divided into boxes with labels such as 'Chemistry', 'Physics', or 'Biology'; when we open the Chemistry box we find smaller boxes 'Organic', 'Inorganic', or 'Physical'; and on opening them, we find even smaller boxes. But is this the way science should be carried out? Are there real boundaries between Chemistry, Biology, and Physics? And do scientists always set out with a well-defined question and proceed to solve it in a logical manner? In this article I explore some of these questions, using our own experience of accidental discoveries, wrong conclusions, and outrageous experiments in biological NMR as a case history.

In the early-1980s we began using conventional solution-state NMR experiments to study the metabolism of small molecules in live bacteria. We teach undergraduates that only clear solutions of pure compounds should ever be placed into an NMR spectrometer, but adding ^{13}C- or deuterium-labelled substrates to suspensions of live cells we were easily able to see the biochemistry that interested us[1–4] without interference from the hundreds of natural-abundance components present within the cell. These background signals must have been present, but in most species they were so small and spread out over the spectrum that they were invisible, or at least insignificant.

However, in 1986 my student Glenn Barnard tried to study methanol oxidation in *Methylobacterium* AM1 and *M. extorquens* and found that his spectra were always dominated by natural abundance signals from within the cell – an example is shown in Figure 1. This spectrum, from a culture grown on unlabelled methanol as sole carbon source, contains six intense peaks in the 60—100 ppm region that we easily assigned to the disaccharide trehalose, (1), and several other peaks in the same area that clearly belong to some kind of polysaccharide. Another compound gave peaks around 20, 40, 70, and 160 ppm and was present in amounts that varied dramatically from one preparation to another; its identity remained a mystery for a few weeks until we accidentally came across a paper[5] describing the solid-state NMR spectrum of polyhydroxybutyrate (PHB), (2). The shifts matched almost perfectly, indicating that PHB was the mysterious and variable component. Further NMR experiments on cell fractions rapidly confirmed our assignments, the trehalose and PHB being found in quite separate parts of the cell as expected.[6]



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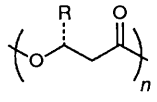

(2) R = Me (PHB)
(3) R = Et (PHV)

PHB is an energy storage polymer of molecular weight around a million.[7] Many bacteria make PHB and store it in the form of sub-micron sized granules when they lack the complete range of nutrients required for cell division but have a generous supply of carbon. If the carbon supply fails, then a depolymerase breaks the polymer down to monomer. PHB constitutes up to 90% of the dry weight of some organisms; the isolated polymer, and its co-polymer with hydroxyvalerate [HV, (3)] is a fully biodegradable plastic which is now commercially available. Having stumbled across PHB in these cells, we became fascinated by what we were seeing: NMR was giving us a new window into the biological and physical chemistry of PHB *in vivo*.

2 Observing PHB Biochemistry *in vivo*

Using NMR, we were able to observe the depolymerization process in a suspension of *M.* AM1 incubated in a carbon-free medium (Figure 2): over a period of hours the PHB can be seen to be completely consumed by the cells, while the trehalose remains largely intact.[8] The role of trehalose is probably osmotic – it is clearly *not* a readily available energy store.

The sensitivity of our current spectrometers is not quite sufficient to allow rapid assay of cultures at viable cell concentrations so these spectra were actually obtained by taking samples from cultures and concentrating them roughly three-fold by gentle centrifugation before NMR assay. Using the same technique it is possible to observe polymer synthesis. For example, we have recently been looking at the incorporation of 3-[^{13}C]-propionate into poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by the commercial and PS-1 strains of *Alcaligenes eutrophus*. The commercial strain rapidly degrades propionate to acetate by loss of C_1 and so should incorporate two labels into both HB and HV if the proposed scheme in Figure 3 is correct.[9] Figure 4 shows part of the spectra of whole cells which have been incubated with labelled propionate and unlabelled glucose. As

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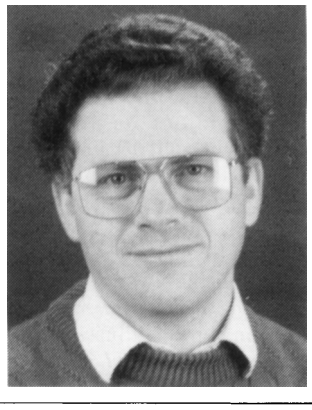

† Dedicated to Professor Ralph Raphael with warm thanks for his encouragement and open-minded interpretation of 'Chemistry'.

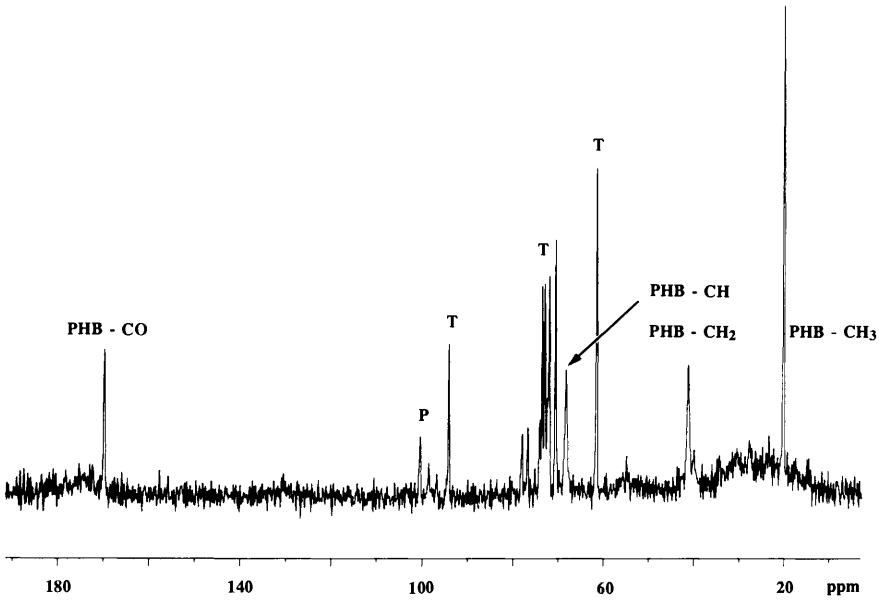

Figure 1 Natural abundance 100 MHz ^{13}C NMR spectrum of a suspension of *Methylobacterium* AM1 cells grown on methanol as sole carbon source. 4000 transients were acquired with 5 s recycle time. **T** marks signals from trehalose and **P** marks polysaccharide signals. (Reproduced by permission from *FEMS Microbiology Reviews*, 1992, **103**, 273.)

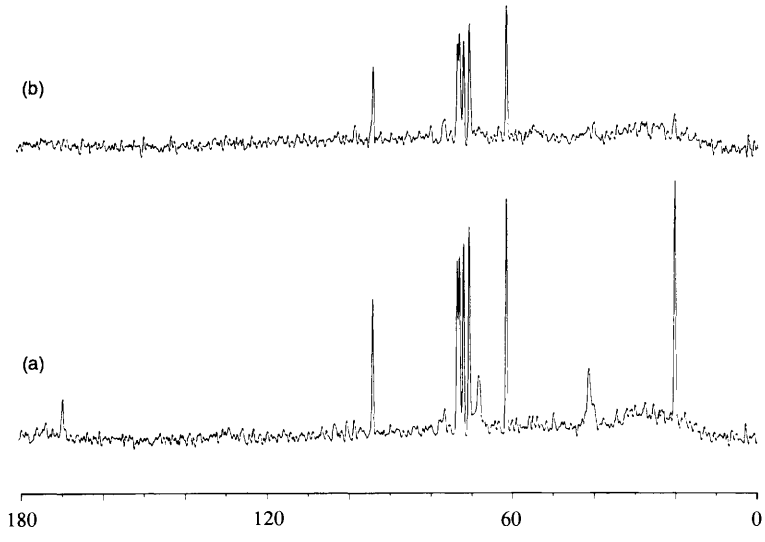

Figure 2 The effect of C_1-starvation on a suspension of *M.* AM1 cells resuspended in minimal medium containing no carbon source. (a) The spectrum immediately after suspension; (b) spectrum after 24 h incubation. 4000 transients were acquired for each spectrum, with a recycle time of 1.56 s.
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expected, in the commercial strain just four positions are effectively labelled [Figure 4(a)]. The PS-1 mutant, which is almost incapable of degrading propionate to acetate, labels only the methyl carbon in the HV units [Figure 4(b)], all the C_2-units in HB and HV being derived from acetate which originates as unlabelled glucose.

Using NMR, it is possible to monitor polymer production in live cells in a more subtle way. For example a switch of carbon source, or of labelling pattern in the same substrate, during polymer accumulation allows us to generate molecules which are effectively labelled in the time domain, *i.e.* newly synthesized material is labelled differently from older material. This opens the possibility in future of looking, for example, at whether the depolymerase attacks the oldest or newest polymer first, or indeed whether such attack is random.

3 Granule Morphology *in vivo*

Solids do not usually give high resolution spectra when solution-state techniques are applied; their signals are too broad to be observed by such simple methods.[10,11] In 1986 the conventional wisdom was that PHB in granules is a crystalline solid so the observation of PHB in live cells using solution-state NMR was very surprising. Indeed, we were successful only because we were ignorant of conventional wisdom: experts in PHB had not used this simple approach because they 'knew' it would not work. Using solution-state NMR techniques we were able to show that the bulk of PHB in granules is not crystalline, and indeed is mobile on a timescale of around 10^{-7} s.[12] Variable temperature and relaxation experiments allowed us to characterize the

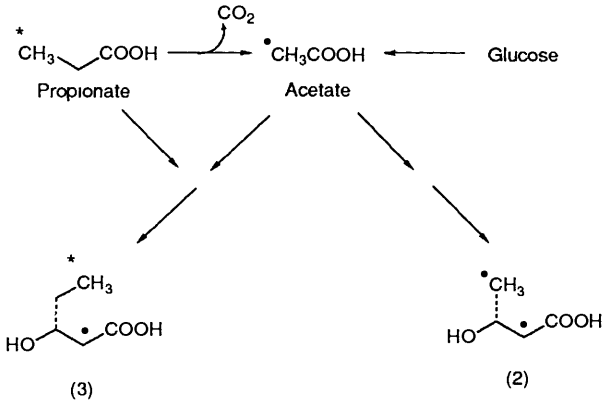

Figure 3 Proposed pathway for incorporation of 3-[^{13}C]-propionate into PHB HV * indicates a ^{13}C-label incorporated directly from propionate while ● indicates a propionate-derived label incorporated *via* acetate

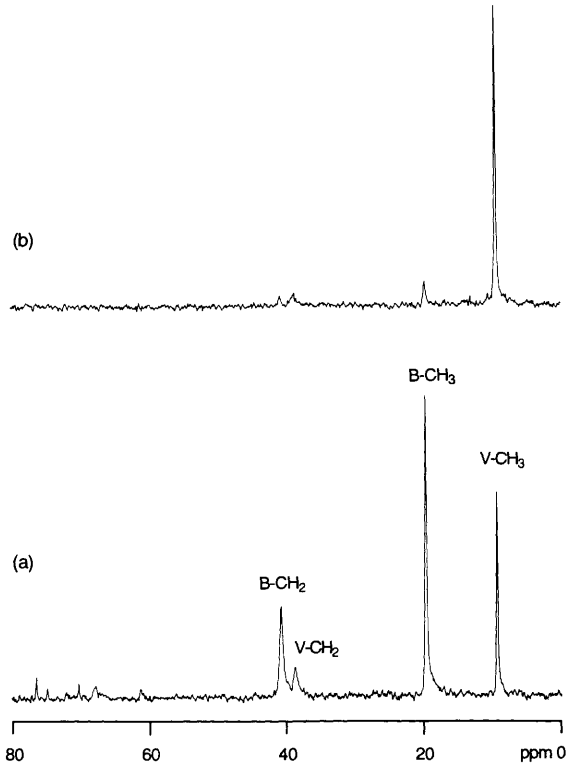

Figure 4 Incorporation of 3-[^{13}C]-propionate into PHB–HV co-polymers by (a) the commercial and (b) PS-1 strains of *A eutrophus* (Reproduced by permission from *FEMS Microbiology Reviews*, 1992, **103**, 273)

motional properties of PHB within the granule and show that they were similar to those of bulk elastomeric (rubbery) polymers above their glass transition temperature, T_g,[13] the glass transition temperature is difficult to define precisely, but it marks the lower temperature limit of rapid, long-range molecular motion

It had long been recognized that PHB granules have paradoxical properties For example, their ability to be rapidly degraded by depolymerases is hard to understand if the polymer is crystalline, even more curiously, mild treatments such as centrifugation lead to rapid and irreversible loss of degradability, even though the PHB molecule is rather robust and remains intact after granule inactivation We realised that many of these problems would disappear if the native granule contains mobile amorphous polymer and if the inactivation is due to irreversible crystallization This idea was confirmed by showing that many of the treatments which lead to biological inactivation of the granule also lead to loss of the high resolution NMR spectrum and the simultaneous appearance of the characteristic *X*-ray powder diffraction pattern of crystalline PHB [12 14]

Our results resolved some of the problems of PHB granules, while at the same time apparently raising new ones how does the cell prevent PHB crystallization *in vivo* and what triggers crystallization when the granules are isolated? For some years, we and others searched for small organic components that might act as plasticizers or nucleation inhibitors within the granule, freeze-drying and other results suggested that water also plays a role [12 14] All this searching was in vain – nobody has been able to find any positive evidence at all Recent spectroscopic experiments in Cambridge (D M Horowitz, unpublished) on highly purified but enzymically active granules show no trace of any plasticizer, while the sedimentation behaviour of these granules on sucrose gradients corresponds precisely to that expected for the density of isolated, dry amorphous polymer, so the water content must be minimal Perhaps most persuasively, the fact that apparently competent granules can be generated in *E coli*[15 16] and even in green plants[17] by transfer only of the genes required for PHB synthesis suggests a much less sophisticated mechanism than plasticization by any specific molecule

We now believe that the explanation is much simpler and lies in the physics of crystallization Organ and Barham had previously found that the maximum rate of spontaneous crystal nucleation for pure isolated PHB is less than 10^2 events mm^{-3} s^{-1} [18] The volume of an individual native granule is of the order of 10^{-9}—10^{-10} mm^3, so the nucleation rate *per granule* is rather less than 10^{-7} events s^{-1} Provided that crystallization of one granule does not trigger crystallization of a neighbour (Figure 5), the average lifetime of a native granule before crystallization will exceed 10^7 s, *i e* months or even years If this explanation is correct, then we were all looking at the 'problem' in the wrong way cells are able to store PHB in a thermodynamically unfavourable state simply because the crystallization kinetics are so slow The *in vivo* state is under kinetic, not thermodynamic control This state is highly mobile, with many of the properties of a supercooled liquid

When granules are subjected to centrifugation or other indignity, they readily coalesce into larger masses Now, single nucleation events occurring with the same *intrinsic* nucleation and propagation rate constants will crystallize a much larger amount of material and lead to an increase in the apparent crystallization rate (Figure 5)

If this model is correct, then PHB in the cell should have the same T_g as supercooled isolated PHB which has not been allowed time to crystallize Our preliminary Differential Scanning Calorimetry (DSC) results (J Clauss and D M Horowitz, unpublished) appear to confirm this prediction Normally the DSC plot of an aqueous suspension of whole cells around 0 °C is dominated by the ice–water transition, but use of a 2 1 glycerol/water mixture shifts this transition to around − 40 °C We can then see the more interesting, but usually hidden, T_g for PHB–HV(90 10) co-polymer of 0 ± 2 °C Precisely the same transition is observed for dry cells and for isolated co-polymer which has been melted and then quenched rapidly In whole wet cells, no transitions are observed below 0 °C These results indicate that no specific plasticizer is required and seem to confirm our kinetic explanation for polymer mobility in the granule de Koning and Lemstra independently came to these same conclusions and devised a similar DSC test [19] Furthermore, they realized that any damage done to the surface coating of the granule will allow heterogeneous nucleation, *i e* crystallization induced by external molecules other than PHB, and further accelerate crystallization [19]

In retrospect, and for reasons that are still unclear, the NMR results[12 14] appear to have misled us about the *in vivo* T_g, and the possible role of water Nevertheless, it appears that, after many twists, turns and false trails we now have a reasonable working model of the granule interior, and that biology has found a simple solution relying on the intrinsic physics of PHB

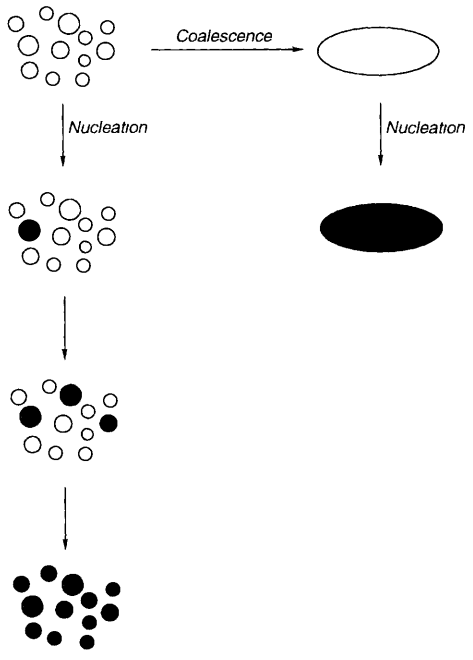

Figure 5 Nucleation model demonstrating that coalescence of native granules by physical treatments such as centrifugation can lead to apparent acceleration of crystallization

Fortunately, one of the false trails led us in an unexpected but fruitful direction – spiders' webs

4 Spiders' Webs

Just about the time we were convinced that water is involved in plasticizing PHB, Vollrath and Edmonds published a paper on the role of water in maintaining elasticity in spiders' webs [20] Over 400 million years of evolution have ensured that spider silk which is predominantly protein – has engineering characteristics that are exceptional even compared to the most advanced man-made materials, one example is the extraordinary elasticity of the coated capture threads in the web of the common garden spider *Araneus diadematus* [21] This spiral capture thread stretches by up to 400 percent when absorbing the momentum of an insect flying into it, whereas the structural thread which the spider builds as a rigid scaffolding is barely elastic at all What is the difference between these threads? What is the significance of the fact that capture threads require a viscid coating to maintain their elasticity? And how can such elasticity be explained at a molecular level?

'Molecular' mechanisms of elasticity envisage that some part of the proteins are flexible and act like springs, while in 'engineering' mechanisms rigid rods slide past each other as in muscle contraction, in each case water is supposed to act as plasticizer or lubricant We realized that our NMR approach should be able to distinguish between these mechanisms because flexible springs should give high resolution solution-state spectra, while rigid rods would not The question then became whether, and how, we could get intact spiders' webs into an NMR spectrometer, and whether we could demonstrate any effects of water

To our delight, Fritz Vollrath, a zoologist in Oxford with little knowledge of NMR and even less of us, agreed to provide web samples in a form suitable for our proposed experiments Each web weighs only around one milligram, and so around 20 capture or structural webs from captive spiders were carefully collected and arranged for spectroscopy as shown in Figure 6, the d_6-benzene capillary provided a lock and a chemical shift reference

A ^{13}C NMR spectrum [Figure 7(a)] of whole capture webs obtained in the absence of any solvent using this capillary assembly showed only the viscid coating, the peaks observed

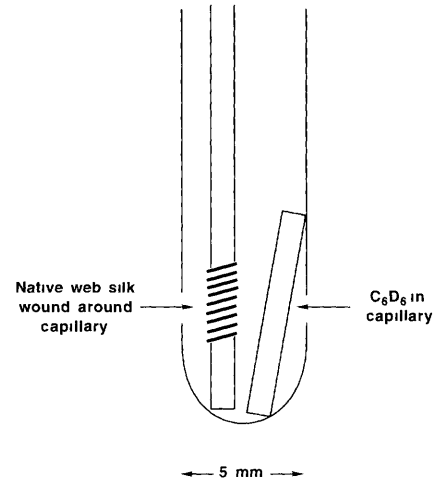

Figure 6 Schematic view of native web assembly and deuterobenzene reference capillary mounted in NMR tube
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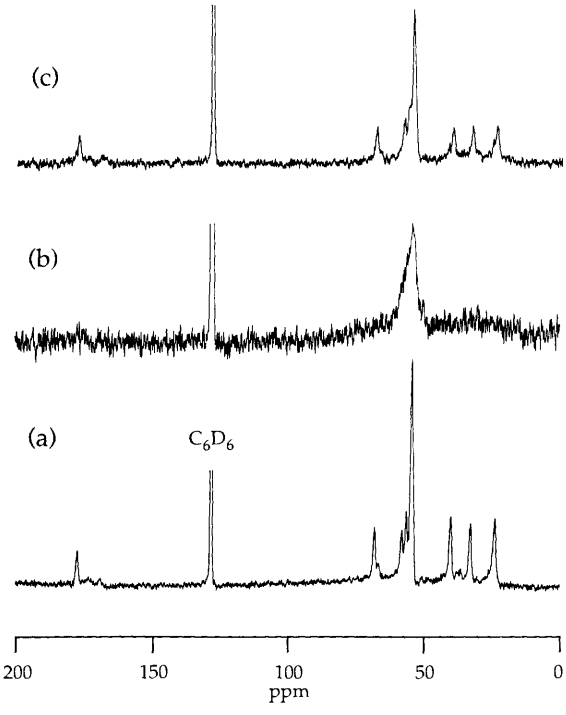

Figure 7 (a) 100 MHz ^{13}C NMR spectrum of 20 whole *Araneus diadematus* capture webs mounted as in Figure 6 only the deuteroben zene (128 ppm) and the small molecule web coating components are visible (b) Spectrum of the same sample after drying over phosphorus pentoxide showing loss of all web peaks apart from the broad Me_3N resonance (c) The spectrum of the same sample after rehydration showing the virtual recovery of all the peaks
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corresponding in shift and relative intensity to the known composition of the water-soluble small-molecule components of this layer [22] The webs were then dried in a vacuum dessicator over phosphorous pentoxide for 24 hours The NMR spectrum [Figure 7(b)] shows complete loss of signal except for a broad hump due to the mobile trimethylamino groups of choline and betaine The webs were then rehydrated over water for 24 hours the NMR spectrum [Figure 7(c)] shows restoration of all peaks loss of water from the viscid layer is reversible This is not trivial spectroscopy each of the spectra in Figure 7 (and Figure 9) is the

result of acquiring over 200 000 transients overnight on a 400 MHz spectrometer

Whole capture webs on the capillary assembly were then thoroughly washed with D_2O to remove the viscid layer and dried over phosphorous pentoxide The dry spiral threads gave no detectable NMR signals, but when they were immersed in D_2O a new spectrum, apparently consistent with that of a protein was obtained Removal of the web from D_2O abolishes the signals, which are therefore not due to dissolved protein

Low sensitivity and sample size in this experiment limited the quality of spectrum obtained We clearly needed to produce ^{13}C-labelled webs Captive spiders generally live on fruit flies but Vollrath's knowledge of spider physiology and psychology allowed him to cut out that particular labelling step hungry and thirsty spiders will drink aqueous solutions quite happily, so he was able to feed them directly over five days with solutions of *U*-^{13}C-glucose or *U*-^{13}C-amino acid mixture (Figure 8) Webs containing only structural threads were collected and wound round glass rods, whole webs containing capture and structural threads were then collected from the same spiders and wound separately from the structural webs

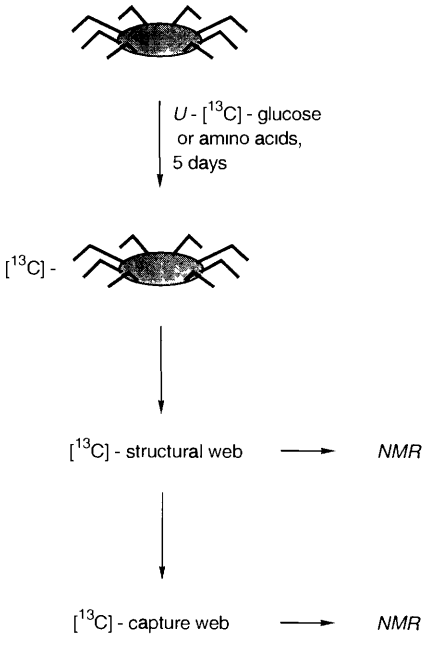

Figure 8 Synthetic scheme for production of ^{13}C-labelled spiders webs

The spectrum of amino acid-labelled capture web after washing and suspension in D_2O [Figure 9(a)] looks very like that of a typical soluble protein, although there are signs that there is better incorporation of label into some residues than others The glucose-labelled webs give similar spectra, but with strong enhancement of signals at 80—85 and 100—110 ppm which appear to belong to the carbohydrate residues of glycoprotein The carbohydrate signals cannot be removed by extensive washing of the webs, implying an intimate attachment rather than weak association The natural abundance spectrum has a comparable intensity to that of a similar weight of the water-soluble yeast alcohol dehydrogenase (MW 150 kDaltons), indicating that we are observing the bulk of the spider silk in these experiments

The crucial observation here is that, in the presence of water, the capture web has regions which are mobile on the NMR timescale The intensity of the capture-web spectrum suggests that a large fraction of the web is visible in these spectra and therefore mobile It follows from this admittedly crude measurement that non-mobile, probably crystalline, regions of the capture threads constitute only a relatively small part of the material The linewidths of the visible signals are consistent with

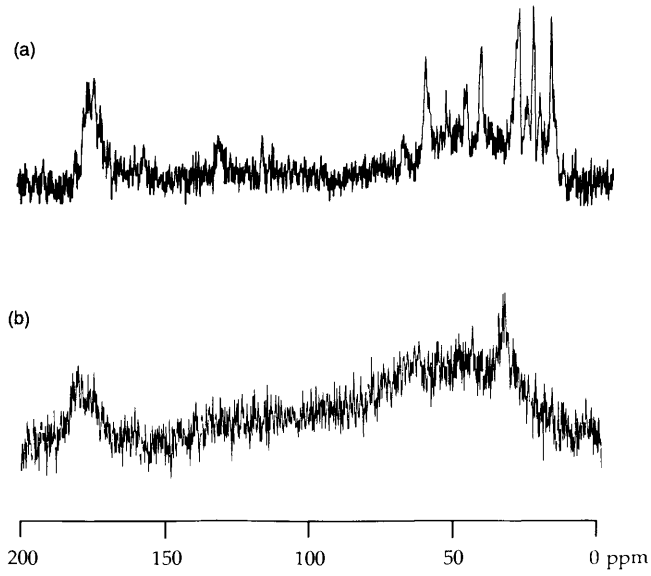

Figure 9 100 MHz ^{13}C NMR spectra of webs from a spider labelled with *U*-^{13}C-amino acids (a) Capture webs suspended in water, (b) structural webs suspended in water
(Reproduced by permission from *Proc R S London Ser B*, 1992, **248** 141)

an elastomer as expected [20] In the absence of water, the mobility of capture-thread protein is greatly reduced and no high-resolution spectrum is obtained Water is therefore acting as a mobilizer or plasticizer for the protein, and the molecular mobility of the capture thread provides the most likely mechanism facilitating thread elasticity One role of the viscid coating is to absorb water from rather dry atmospheres and so provide an aqueous environment for the capture thread, it even provides a source of drinking water for the spider in dry weather [23]

By contrast, structural threads suspended in D_2O give no high resolution signals under the same conditions [Figure 9(b)] They lack either molecular mobility or elasticity, even in water, and clearly have a quite different arrangement at the molecular level It may well be that solid-state experiments of the type described in the following section will throw further light on this

5 Solid-state NMR of Isolated Polymers

Modern ^{13}C NMR techniques allow us to probe both order and mobility in solid isolated polymer samples [10 11 24] In our solid-state work, magic angle spinning (MAS) is always used to generate narrow lines, allowing access to a variety of subtle techniques and parameters We can distinguish crystalline from amorphous – or, more precisely, conformationally ordered from disordered – material by chemical shift differences of some carbons,[8] and mobile from immobile regions by a variety of relaxation and linewidth phenomena To simplify somewhat, nuclei that are able to re-orient within the solid in less than *ca* 10^{-7} s give sharp NMR lines while those that are less mobile than this give very broad lines

The simplest ^{13}C 'one-pulse' experiment detects the most mobile carbons, and so tends to emphasize amorphous regions If we employ cross-polarization from ^1H to ^{13}C, then we detect the least mobile material and so tend to emphasize the crystalline regions Figure 10(a) shows the cross-polarization spectrum of a sample containing 16% HV the signals are almost exclusively due to HB, any HV having been effectively excluded from the crystalline domains By contrast, the 'one-pulse' spectrum [Figure 10(b)] shows that the less ordered regions are enriched in HV, and the HB methyl carbon in these amorphous regions has significantly different chemical shifts from crystalline HB In co-polymers with very high HV content, the chemical shifts of both HB and HV in crystalline regions are significantly changed

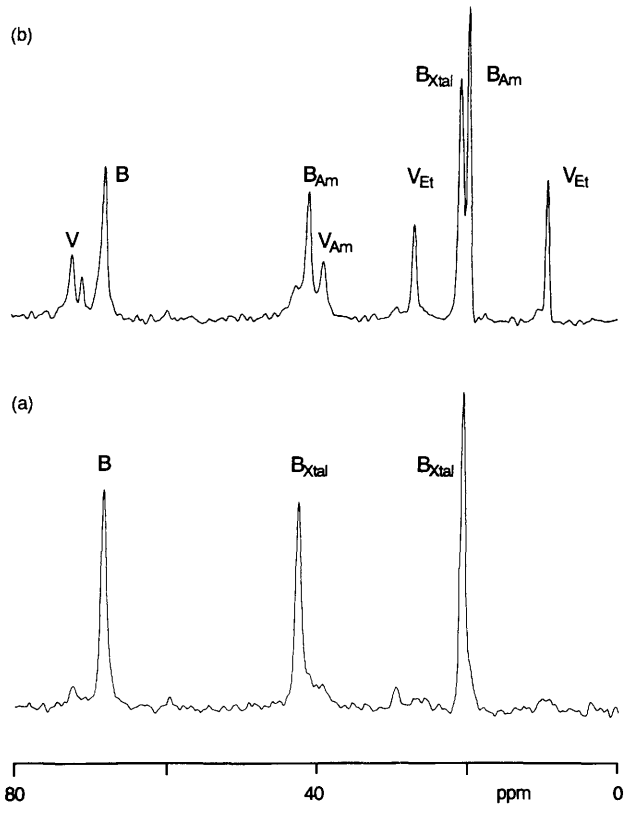

Figure 10 50 MHz spectra at 320 K of a solid sample of poly(HB–HV) containing 16% HV. (a) Cross-polarization spectrum (1 ms contact time; 3 s recycle time) showing that the least mobile fraction contains almost exclusively HB units. (b) 'One-pulse' spectrum (recycle time 1 s) showing concentration of HV signals in amorphous, mobile phase.
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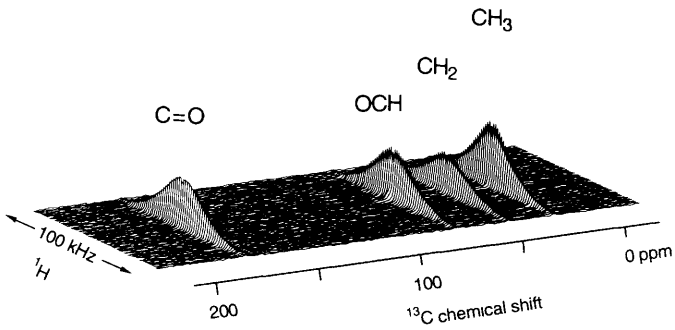

Figure 11 Two-dimensional 75 MHz wideline-separation spectrum of pure isolated PHB at ambient temperature.
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again, indicating the existence of another crystalline phase, this time based on HV.

Two-dimensional NMR techniques open up new types of information.[24] For example it can be arranged that one dimension contains conventional ^{13}C shifts (and therefore structural information), while the other displays the linewidth (and hence mobility) of the protons providing cross-polarization for each ^{13}C. This allows us to search for any differences in the mobility of the crystalline and amorphous regions as a function, for example, of temperature. Thus the spectrum in Figure 11 shows pure PHB at ambient temperature. There is no detectable highly mobile region. The sample is around 70% crystalline, but the disordered chains between crystalline domains are so short and tightly constrained that they too show little mobility on this NMR timescale.

By contrast a co-polymer sample containing 29% HV clearly shows at 340 K the presence of immobile crystalline HB regions and more mobile amorphous regions (Figure 12). Finally, by irradiation of the signals in one environment and observing the effect on signals in other environments, one can measure the rates of spin diffusion between amorphous and crystalline regions to get estimates of domain sizes.[24]

6 Conclusions

The opening of this article posed questions about the boundaries of science and about the way science is done. I would claim that all our work described here is Chemistry, in the sense that it looks for understanding and explanations at the molecular level. But it was only possible because the techniques of physics, materials science, microbiology, and behavioural psychology were available and exploited in pursuit of that molecular understanding. Furthermore, it is genetic engineering combined with the physics of crystallization that have provided the latest insight into the physical chemistry of the PHB granule *in vivo* and which may lead to our being able to reproduce this intriguing state in the laboratory.

It is only through familiarity with such a range of sciences, scientists, and their differing vocabularies, that one can hope to make progress in such an area – much of the fascination is precisely in the challenge of understanding and choosing the problems which are presented by other disciplines and then solving them using a chemical approach. Unfortunately there are strong counter-pressures on scientists in all disciplines to specialize in safe and well-defined areas, and to read and publish in inward-looking journals.

Our first breakthrough in understanding PHB granules in 1986 came about accidentally, and we were lucky to be able to switch our attention to this more exciting project without formality. Nobody who 'knew' that PHB is crystalline *in vivo* would have attempted our experiment, and certainly nobody could have got a grant to do the work until after the crucial results had been obtained. Our spider work only happened because in 1989 we mistakenly believed that water was a plasticizer for PHB and so we drew an incorrect analogy. The experiments on spiders webs were deliberate, and reasonably well-planned, but would probably have looked even less grant-worthy than the granule work. They were only possible because

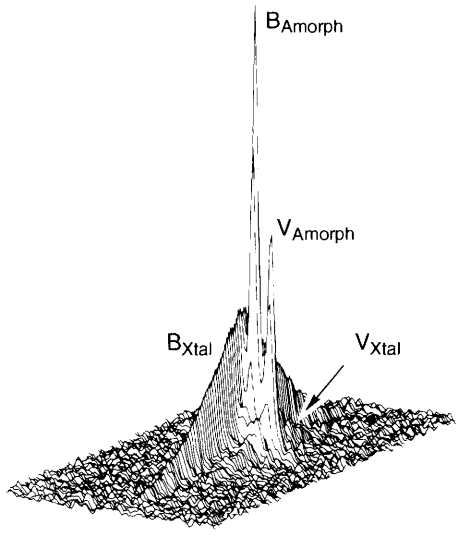

Figure 12 The methylene region of a two-dimensional 75 MHz wideline-separation spectrum at 340 K of PHB–HV co-polymer containing 29% HV.
(J. Clauss, unpublished spectrum.)

we could afford to divert small amounts of labelled precursors from another project, and because our offbeat approach was welcomed by Vollrath

There is, then, another dimension to how we do science apart from the narrow *versus* wide perspective, and that is how we organize and fund it one can choose a big problem, lay out a master plan for solving it, obtain the funding and people, and solve it, or one can set out to explore an interesting but superficially trivial by-way while always keeping one's eyes, and mind, open The first approach is clearly a vital component of any system – in the right hands it can lead to outstanding and exciting science The second is often more difficult to justify when short-term thinking and target-oriented research are fashionable, and the danger of a fully grant-led culture is that the second approach becomes impossible, except perhaps within the umbrella of a large successful group operating the first approach However, it is arguable that this less deterministic approach gives more room for individual expression and development by the person actually doing the work, and has a greater potential to make completely unexpected breakthroughs in unpredictable areas We certainly feel it can be more fun

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