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Introductory Remarks

R. J. P. Williams

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Phil. Trans. R. Soc. Lond. B **289**, 381–394 (1980) [381]
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Introductory remarks

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In this introduction I wish to analyse the problems that we face when using n.m.r. to tackle biological states *in vivo*. I shall refer to high resolution n.m.r. only, since the second half of the Discussion is devoted to low field methods. When referring to *in vivo* systems I shall include isolated whole cell systems, excised whole organs, and whole body studies, but I shall not discuss extracted chemical solutions. Studies of extracted solutions proceed by conventional means. I shall use a range of observations for illustrative purposes; most of these observations refer to work in which I have played a part at Oxford. The people who have been concerned in initiating this work are Dr I. D. Campbell, Dr C. M. Dobson, Dr P. E. Wright, Dr B. Levine, Dr A. Daniels and Dr R. G. Ratcliffe. In this symposium Dr Campbell and Dr Dobson will report in detail on some of their independent work.

I wish first to give a quick survey of nuclear magnetic resonance spectroscopy (n.m.r.) for those who wish to read the following papers but have no knowledge of the method. N.m.r. is one kind of absorption spectroscopy. An n.m.r. spectrum is therefore a plot of absorption against energy; each different chemical gives rise to its own n.m.r. spectrum. The absorption of energy is by magnetic nuclei orientated in a magnetic field and many different nuclei can be studied. A compound such as adenosine triphosphate (ATP) has three different n.m.r. spectra due to the nuclei ^{1}H, ^{13}C, ^{31}P. The intensity of each line is proportional to the number of atoms of exactly the same kind. Fine structure on the lines of a given nucleus depends upon the neighbouring nuclei and upon their magnetic moments. Thus, ATP has three phosphorus atoms and gives three ^{31}P signals (figure 1). The first and last, α and γ, have one phosphorus neighbour and the middle, β, has two such neighbours. The splitting pattern of the lines is two doublets and one triplet since each phosphorus nucleus has a spin $s = \frac{1}{2}$ which couples with its immediate neighbour and every line that couples to n equivalent $s = \frac{1}{2}$ nuclei is split into $(n+1)$ components. As the α, β, and γ phosphorus atoms are in different chemical environments their transitions are of different energy. It is seen that any n.m.r. spectrum is a good molecular fingerprint. The area under each signal is the same as the integrated intensity of absorption and is strictly proportional to the number of atoms of one kind. However, the intensity is not the same for different types of nuclei. N.m.r. is at its most sensitive in proton studies, both through the abundance of the isotope ^{1}H and the size of its nuclear gyromagnetic constant. Some elements have magnetic nuclei only through low abundance isotopes, e.g. ^{13}C and ^{17}O; their most common nuclei, e.g. ^{12}C, ^{16}O, are diamagnetic and do not give n.m.r. spectra. N.m.r. is not a very sensitive method, especially for heavy atoms. This symposium will be largely limited to ^{1}H, ^{13}C and ^{31}P spectroscopy.

Apart from the energy–intensity relations in n.m.r. spectra it is also possible to get information about the dynamics of molecular processes from the measurement of relaxation times, i.e. the rates of energy distribution after absorption. There are two relaxation times, T_1 and T_2,

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the lattice relaxation and the spin–spin relaxation, respectively. Relaxation times of small, free molecules, which tumble very rapidly, are recognizably very long; it is for this reason that small, free molecules give readily observable lines in the study of biological objects, while the broad line spectra of large molecules are usually lost. There are developing techniques for the study of larger, slowly tumbling, assemblies, e.g. membranes and bones, but in this symposium only one paper, that of Griffin, refers to these methods. It is also probable that the use of deuterium n.m.r. will greatly aid the study of *in vivo* membranes (Smith *et al.* 1977). Technical details of the methods of study of the relaxation times and a full explanation of n.m.r. spectra are given in Dwek (1973). In n.m.r. spectroscopy, we receive information from five quantities, namely the chemical shift or energy, δ, the intensity, the splitting pattern of a line, and the relaxation times, T_1 and T_2.

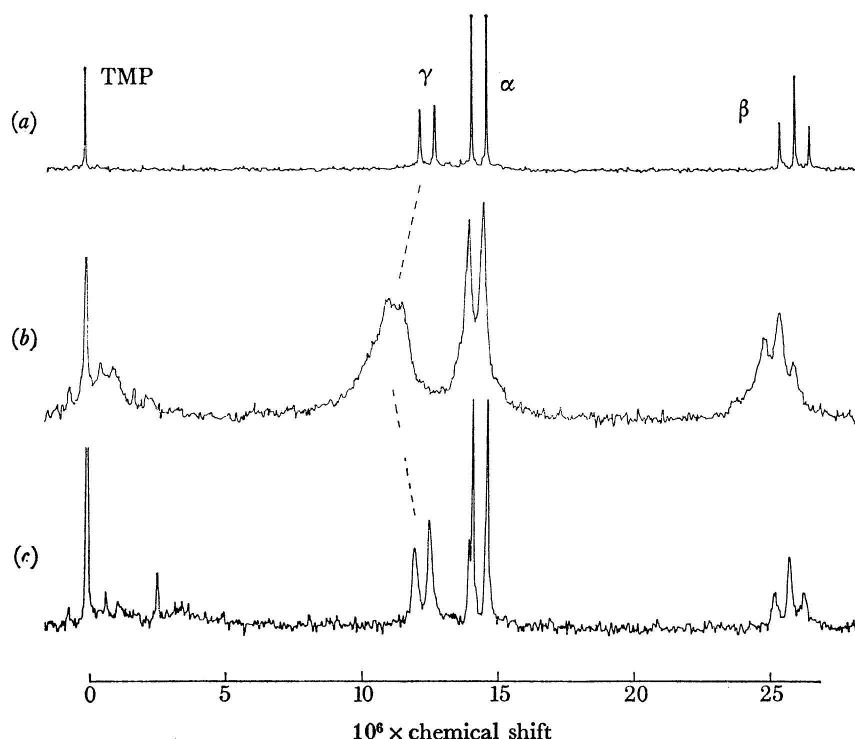

FIGURE 1. The ^{31}P n.m.r. spectrum of adenosine triphosphate (ATP), showing (*a*) the α, β and γ peaks of free ATP relative to the peak of trimethylphosphate (TMP), (*b*) the ^{31}P peaks seen in the chromaffin granules and (*c*) the ^{31}P peaks seen in the lysed granules. Note that in (*b*) there are weak extra peaks that have not been explained. Spectra exactly like (*b*) were obtained from whole rat adrenal glands. (From Daniels *et al.* (1974).)

A very brief history of the early work on intact systems will be used to illustrate these initial points.

The first significant observations that I have traced on intact unicellular biological systems by means of high resolution n.m.r. methods are the studies by means of ^{13}C n.m.r. of yeast metabolites by Eakin *et al.* (1972), and a similar series of observations by ^{31}P n.m.r. by Moon & Richards (1973), who studied the erythrocyte. Much new work on unicellular organisms will be described in this symposium by Campbell and by Shulman and their coworkers. My own interest in this field was stimulated by working with Dr I. D. Campbell and Professor C. M. Dobson on differential relaxation rates of proton n.m.r. signals from within proteins of different

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internal mobility. This led me, in 1973, to put some lupin seeds (from my garden in Oxford) into an n.m.r. tube and to examine their high resolution proton n.m.r. spectrum. The experiment was conducted in order to see if there were *mobile* molecules in the seeds. (The idea of doing such an experiment was greeted with considerable scepticism.) We were surprised to see any spectrum at all and were more than satisfied to find that the only sharp signals were from the unsaturated *mobile* fatty acids of the store of fat (oil) in the seed (Daniels *et al.* 1977*a*).

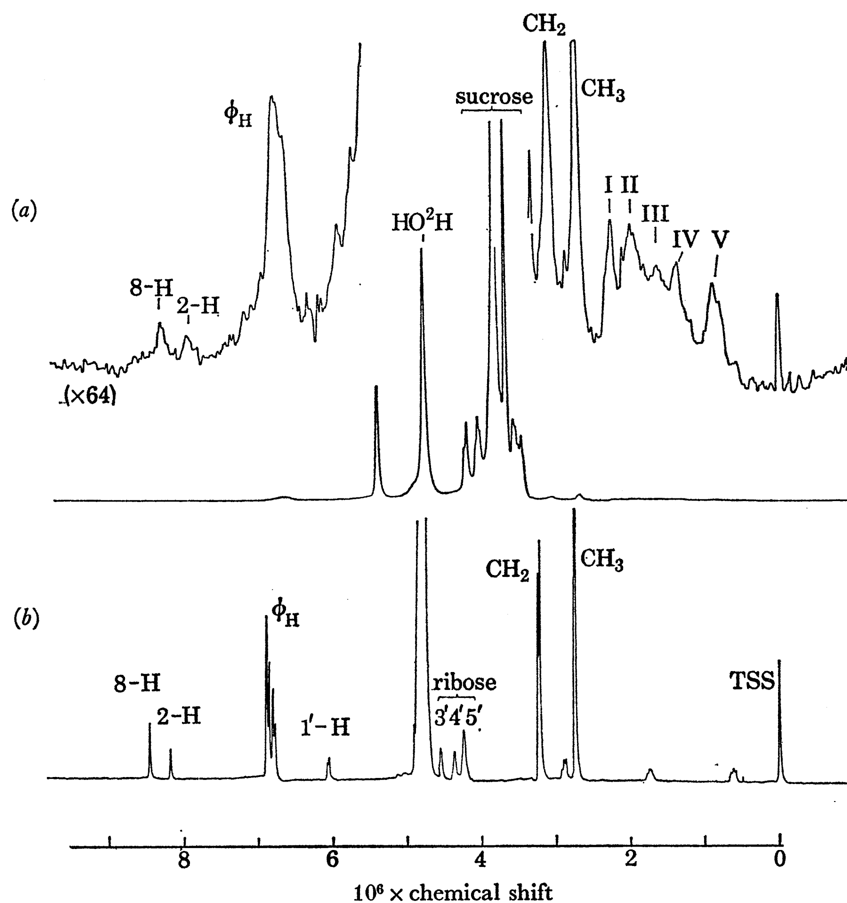

FIGURE 2. The ^{1}H n.m.r. spectrum of (*a*) the chromaffin granule, which is exactly like the spectrum of whole rat adrenal medulla; the very sharp lines to the left of the CH_2 and CH_3 peaks are of released adrenalin; (*b*) a 4:1 mixture of adrenalin and ATP; the 8-H, 2-H, and ribose peaks, 1′-H, ribose 3′, 4′, 5′, are from ATP; the peak marked ϕ_H is from the aromatic ring of adrenalin. In (*a*) protein peaks are marked with roman letters.

We also looked briefly at the ^{13}C spectrum of the seeds and made the observation that again only the oil store was visible. We immediately looked at other parts of plants and again saw some signals typical of small mobile molecules. All these experiments show that in n.m.r. experiments *in vivo* one sees only a *very* limited number of chemicals. At first it was supposed that *only* the *small* mobile molecules could be visualized.

At about that time (1973) I started on a joint venture within the Oxford Enzyme Group, and especially with Dr Radda, to study the adrenal gland, a very complicated multicellular organ. This marked the beginning of our whole organ n.m.r. studies. The glands that we studied were from the rat and, as they were almost exactly the same size as a small pea-seed and just went into an n.m.r. tube, we decided to run their spectra. These experiments showed

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only H, C and P spectra (by ^{1}H, ^{13}C and ^{31}P n.m.r.) of the internal content of adrenalin, ATP and a protein of the chromaffin granules, and almost nothing from the whole of the rest of the cell population (figures 1, 2). This was a complete surprise. The spectra make again the central point that n.m.r., studied in the conventional way, sees only a *very* limited set of (mobile) molecules. (A seed does not seem to have any mobile chemicals and this was the reason for the laughter when I started to look at seeds.) A literature search in 1974 showed, in fact, that this point had been well taken, independently, by another research group under Dr Schaeffer, who conducted an outstanding series of experiments on plants (Schaeffer & Stejskal 1974; Schaeffer *et al.* 1975; Schaeffer *et al.* 1978). The plants were grown in $^{12}CO_2$ initially but at a given stage of development growth was switched to a $^{13}CO_2$-enriched environment. Analysis of the plant organs now followed the time course of incorporation of ^{13}C into different mobile oil molecules. I understand that the work has been under continuing development at Monsanto and regret that we could not persuade Dr Schaeffer to report on the work here. The study of unicellular systems, seeds, plants and the adrenal gland opened a vast potential field for n.m.r. of the study of intact biological systems. I shall now leave the historical aspects and concentrate upon the problems inherent in this type of study and its potential.

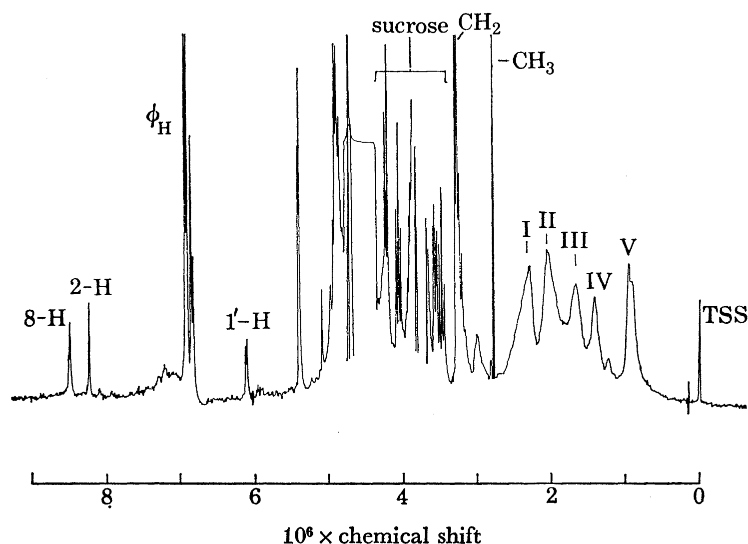

FIGURE 3. The ^{1}H spectrum of the lysed chromaffin granules, showing how the peaks sharpen in free solution (compare with figure 2).

INFORMATION FROM N.M.R. STUDIES

In the above, I have stressed the need for mobility if *in vivo* analysis is to be done by n.m.r. (T_1 and T_2 are long for such molecules.) Quantitative analysis is one major use to which n.m.r. could be put in *in vivo* studies; factors affecting analysis will become obvious below. N.m.r. is not just any analytical method at the level of revealing the number of atoms of a given kind, however, but it can show, also, the particular combination of the atoms present, the structure of individual compounds and the way in which different component molecules are assembled in an organized fashion, and it can give rates of exchange of molecules between different situations. In principle, it can also be used to map chemicals in biological space. I will now look at some of these features of the method with the aid of chosen examples from work with which I have been associated.

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(a) *Analytical sensitivity*

Analysis by n.m.r. is notoriously poor. It is, therefore, essential that quantitative chemical analyses of extracts are provided together with n.m.r. observations on *in vivo* systems. This follows from the low sensitivity of n.m.r. as a method: the detection limit, which is rarely reached, is around 10^{-4} M and the analytical limit is around 10^{-3} M. There is a further loss of information when the relaxation times are such that no signals, or only very broad signals, are seen. Broad lines can arise simply because some molecules are large (slow molecular tumbling), or through the presence of slow electron-relaxation reagents (paramagnetic effects), or through broadening of resonances, even of small molecules, due to slow exchange between bound and unbound forms. It is clear that the size of the molecule is not the real limiting factor on analysis. Indeed, we have seen, by proton and phosphorous n.m.r., large proteins in the adrenal gland (chromagranin A) and in eggs (phosvitin) (figures 2, 3), and many small molecules that are present have not been seen. Even these simple statements about signal intensities and analysis hide a further problem, that of the inhomogeneity of magnetic susceptibility across the sample. There is a general broadening of resonances due to the inhomogeneous field since this produces many different, closely related energy transitions. A given transition is spread into a multiplicity of transitions. The problems of the effects of varying motion, varying concentration of paramagnetic ions, varying bulk susceptibility and a variety of exchange processes must all be tackled at a fundamental level unless the n.m.r. observations are backed up by a series of analyses of micro-extracts. Unfortunately, in a biological system there will be a great variety of components with every kind of line width. It is surprising, in fact, that n.m.r. measurements on whole tissues are as successful as they are. One difficulty we have encountered refers to the three ^{31}P resonances of ATP in chromaffin granules. These resonances do not seem to have accurately equal intensities, even when we can see coupling patterns. Another difficulty concerns, for example, the marked effect of bone in animals on line shapes (see below).

Some of the technical problems of uncovering resonances of molecules of different mobility and often very different relaxation times, in whole organs, were solved by resort to 'tricks', especially the use of pulse methods developed by Campbell *et al.* (1975), learnt in the study of protein chemistry. Daniels *et al.* (1976) and Brown & Campbell (1976) showed that chosen pulse sequences make it possible to find the sharp resonances of small molecules against a broad general background. The method has been greatly used by Campbell and his collaborators in the study of the red cell. A major step forward would be the further development of techniques for the differential adjustment of line shape (see the paper by Griffin in this symposium). Again it is necessary to add a cautionary word. While these pulse methods allow us to find the sharp lines, we must be very careful if we attempt to use these subspectra in analysis.

The problem of quantitative analysis is then bedevilled at higher concentration, 10^{-3} M, not so much by lack of sensitivity as by change of line shape (and energy) with environment. This problem is not found in other forms of spectroscopy, but n.m.r. lines are particularly sensitive to changes in viscosity, to the association of species, to inhomogeneity of field (susceptibility) and to small amounts of certain impurities. This means that a full integration over a line, and not just observation at one frequency, which is the common procedure in other forms of spectroscopy, is needed. Given these restrictions, a very thorough analysis of

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the theory of line shapes has to be undertaken. Living systems have some structures of markedly different susceptibility to water and line shapes in the region of bones or shells may be most unusual. Dr R. G. Ratcliffe at Oxford in undertaking a detailed study of these problems.

(*b*) *Chemical state*

As stated already, it is not enough to be able to recognize a chemical: we should like to be able to define the chemical form that it is in. There are many chemical difficulties. Consider figure 4. Let us suppose that we can observe the total ATP in one pool, as proved by chemical analysis, that there are no field inhomogeneities and that paramagnetic effects are absent.

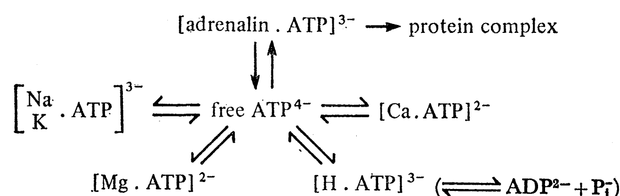

Figure 4. Some of the multiple equilibria in which ATP is involved within a single compartment, here the chromaffin granule. Each complex shifts the lines in figure 1. In biological systems, many such equilibria are present and n.m.r. may only see an averaged signal over all. Additionally, many different compartments may be present.

Of the reactions in figure 4, the observation of separate signals for ATP, ADP and P_i now gives their separate total concentrations. (Note that ADP and P_i undergo *slow* reaction to give ATP. Note, too, that they undergo exchange reactions much as those shown for ATP.) Now the other reactions in the figure must be in fast exchange, for otherwise we could not have seen, say, the protein-bound ATP fraction. With these advantageous assumptions, it is clearly still impossible, from the study of the limited chemical shift data, to describe the six equilibria (there are more) shown. Thus, to understand the signal observed, we must know the analytical concentration of some of the variables, H^+, Mg^{2+} and the binding proteins, independently. Moreover, all these constants depend upon ionic strength. The temptation will be to assume that we can ignore certain of these equilibria and therefore deduce, say, the pH of the solution. Often this will be true, but we must be careful. I shall now illustrate the analysis of these equilibrium problems by means of the chromaffin granule, which has been studied by two groups in Oxford, my own and that of Dr Radda, and more recently by several elsewhere, e.g. Sharp & Richards (1977).

The chromaffin granule

The spectra of this vesicle are shown in figures 1*b* and 2*a*. We show both ^{31}P and ^{1}H signals. The spectra can be observed in whole adrenal glands of rats, slices of adrenal glands of cows and sheep, or on the extracted granules. These spectra are the same. The liberation of the granule contents, followed by chemical analysis, showed that we observed all the ATP, the adrenalin and much of the chromagranin proteins (figures 1–3). We saw no signals from the membrane or, indeed, from any other chemicals except for some weak phosphorus resonances. We were aware of the analytical concentrations of all of the components in the vesicle, including metal ions, especially Ca^{2+} (15 mM). Figure 4 shows the analytical problem. We found that the

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resonance positions for the ATP and the adrenalin were shifted from those of the separated, free molecules, showing that there were intermolecular complexes between protein, ATP, metal ions and adrenalin. In order to study the equilibria shown in the figure, we have studied many interactions between pairs and triplets of the components. There are many different interactions, all in fast exchange. The shift and relaxation n.m.r. data from the chromaffin granules plus the data from model mixtures led us to propose a structure for this vesicle. Although there is not complete agreement in all details (Daniels *et al.* 1974; Daniels 1977), the work of Sharp & Richards (1977) has confirmed most of them. This work and that of Njus *et al.* (1978) illustrates the power of n.m.r. in the discussion of equilibria *in vivo* as opposed to deductions about equilibrium states from experiments *in vitro*.

I now go forward to other information that n.m.r. studies of whole systems can give.

The mobility of components

There were some additional striking observations concerning these spectra. The line shapes and the relaxation times, T_1 and T_2, were different from those of free molecules and we were led to discuss the viscosity of the internal medium of the granule. Finally, we observed not only adrenalin and ATP but also the signals from a protein of molecular mass 80000 (figures 2, 3). This is only possible if the protein is virtually a freely mobile, random coil inside the vesicle inside the gland cell. Thus, we had found **a** functional protein without a fold. Suggestions as to what that function might be have been made. We were also able to suggest, by a probe procedure (Daniels *et al.* 1978) described below in this article, how the metal ions were involved. The above deductions about mobility always require analysis of exchange reactions to be carried out, but the mobility can be found.

In summary, I believe that these experiments show the power of n.m.r. in the study of intact biological tissues in the following ways.

(i) It was proved analytically that the *in vivo* vesicle was identical with the extracted vesicle. No other method could do this.

(ii) The organization and the mobility of the internal contents were found *in vivo*.

(iii) A function of the structure could then be suggested.

We note that our understanding of the vesicle has been considerably increased.

Bioenergetics

Our work failed in one respect, but, fortunately, other members of the Oxford Enzyme Group spotted our mistake. We did not look at the pH equilibrium of the granule. This takes me back to figure 4. While we had considered the shifts on the phosphorus signals of ATP to be due to neighbouring magnetic fields (ring currents), Radda and his associates subsequently (1975) pointed out that they could be due at least in part, to a pH much lower than the one we thought existed (Njus *et al.* 1978). The internal pH is now known to be around 5.7, whereas we used 6.8. Subsequently, this has led Radda to propose that one source of energy for the granule is a proton gradient across the granule membrane. Given the complexity of the equilibrium shown in figure 4, this result could hardly have been obtained without the detailed analysis of the equilibria in extracts and the use of other checks. When this is said, however, we remain with the bonus that n.m.r. of intact cells can be used to observe energy states of

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intact systems and that it can follow changes in such states. These are not just pH gradients. The same equilibria can be used to follow, for example, Mg^{2+} gradients. In fact, the chromaffin granule has a huge Mg^{2+} gradient across its membrane and is, therefore, energized in a quite special way and in the same sense as chloroplasts. The bioenergetics are very complex (figure 5). The major point here, however, is that n.m.r. can be used to study bioenergetics *in vivo*. This includes all kinds of distributions of components and not just ion gradients, e.g., it can be used to study a Donnan potential.

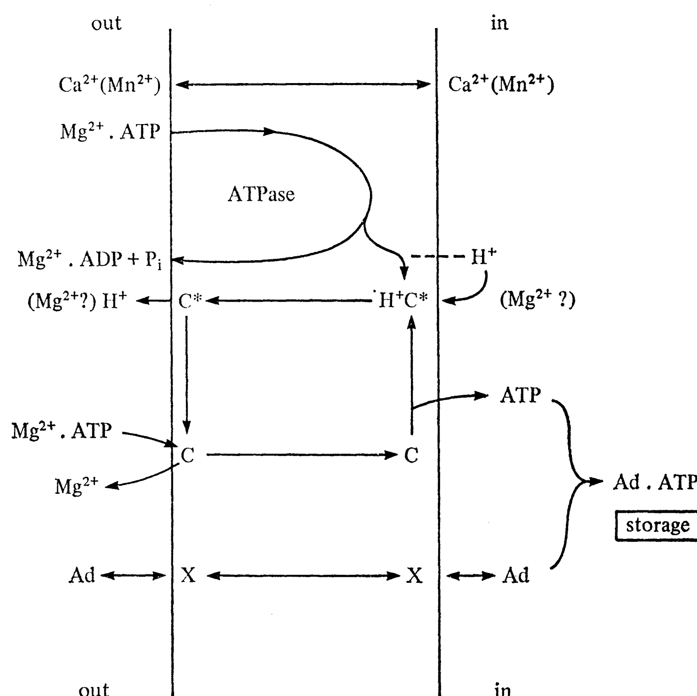

FIGURE 5. The bioenergetic problem posed by the chromaffin granule. There are known gradients of ATP, adrenalin, protons, magnesium and calcium ions, and unknown gradients of chloride, bicarbonate, sodium and potassium ions. There is also a large Donnan potential due to the chromagranin protein. (Ad, adrenalin.)

THE PECULIARITIES OF *IN VIVO* N.M.R. STUDIES

Next we stress what we did not see. In the whole adrenal medulla we saw signals from only one protein, and we saw no signals from any membrane, DNA or RNA species. Of the small molecules present, we saw signals from two. There are hundreds of other species present. This shows that the n.m.r. method, as applied so far, looks at a biological sample through a very narrow window. Just as u.v./visible light can only follow molecules absorbing beyond about 260 nm up to about 1000 nm because of the general u.v. and i.r. absorptions at shorter and longer wavelengths, so n.m.r. sees a very limited set of atoms of one element and nothing from the vast majority. This is simultaneously an enormous advantage, that of simplicity, and an enormous disadvantage, loss of detailed knowledge, and we must hope for improvement in technique to overcome the disadvantages.

Now we know, from independent degradative chemical analysis, that the signals from the adrenal medulla come from the inside of the chromaffin granule. In general, this will not be true of n.m.r. studies, since a biological system has many compartments (mitochondria,

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lysosomes, nuclei, extracellular space), and without some mapping aids we shall see only the sum of the molecules from all these environments. In fact, either one signal representing a sum of the signals from different compartments or a multiplicity of signals could be seen from one chemical that was in these different compartments. Detailed analysis of these situations uses well known n.m.r. methods, but the analysis can be exceedingly complicated (Campbell *et al.* 1978). Again, it is not always possible to find signals that are broadened by exchange. An excellent example of one proven procedure is described in the paper by Dr Truman-Brown in this symposium. However, when the sample volume inspected by n.m.r. is large, we need to be very cautious in interpretation.

Figure 2 gives a clear example of the study of the distribution (compartmentalization) of chemicals, since there are signals from adrenalin inside the vesicle (broad lines) and, nearby, sharp signals of released adrenalin outside the vesicles (Daniels *et al.* 1974). The experiment could be used to follow adrenalin release. Note again in the figure the complications that arise from a variety of line shapes and line positions, here turned to advantage.

Some further examples

In view of the general nature of the possibilities described above, the Oxford Enzyme Group decided to look at a few chosen systems. Those related especially to glycolytic reactions will be described later in the meeting by Campbell, by Radda and by Gadian and Wilkie. My own associates decided to look at storage systems, in addition to the adrenal gland. Table 1 lists some systems of promise. We are studying the variation of the physical state in which molecules are stored *in vivo*. N.m.r., almost alone, can attack this problem. I shall now give a few details about two storage modes.

Table 1. Gland studies by n.m.r.

gland	observations	comment	reference
adrenal medulla	ATP, adrenalin, protein	mobile store	Daniels *et al.* (1974)
adrenal cortex	unsaturated fat, little sterol	mobile fats, relatively immobilized sterols	Daniels *et al.* (1977*a*)
octopus salivary gland	$N(CH_3)_4$-type compounds	mobile molecules in store, no proteins	Daniels *et al.* (1977*b*)
left colleterial gland (cockroach)	aromatic resonances of various phenolic glucosides	*mobile* phenol glucosides and tyrosine rich proteins	Levine *et al.* (1979)
insect haemolymph	phenolic compounds and tyrosine-containing proteins	proteins appear to have little tertiary fold	Egmond *et al.* (1979)
seeds, young shoots etc.	unsaturated fat, phosphorus (phytic acid)?	mobility increase with development of the seed	R. G. Ratcliffe (unpublished)
egg (avian)	phosvitin storage of phosphorus	mobile protein	Krebs & Williams (1977)

An example from insect physiology

The first problem is one of insect physiology. The work was carried out in collaboration with Dr B. A. Levine and Dr R. N. Pau of the Zoology Department, Oxford. Much as the sap of a plant must transfer phenols for the production of chitin and the healing of wounds so the lymph of an insect must carry phenols for the production of the cuticle. A description of the chemistry of the production of these chemicals by insects is given by Brunet (1965). We can put larvae directly into the n.m.r. tube (whole body n.m.r.) and observe the mobile molecules

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in their haemolymph. In this way we observe phenol-containing molecules, and we should be able to follow the changes of concentration of these molecules during the life cycle of the insect. (A very similar possibility arises in plant stems.) Now these transported phenols are to be used to form a material called sclerotin, in which the phenols cross-link special proteins in the hard case of the insect egg coat. There is a gland, the left colleterial gland (we study the cockroach), that produces and stores these proteins together with phenol glucosides. The right colleterial gland produces the enzyme for hydrolysing the glucosides in the blood stream. The proteins have been described by Pau *et al.* (1971) and are notably very rich in tyrosine. The n.m.r. spectrum of the gland shows that the phenol glycosides are free, small molecules and that the protein is highly mobile and has little tertiary structure. The amino acid composition shows that the proteins are rich in glycine as well as tyrosine.

Examination of the proton n.m.r. spectrum shows that all the lines are sharp, the fine structure can be seen, including that on the tyrosines, and that all the lines are virtually unshifted from the positions in a random coil spectrum. The overall impression of this gland is that its tyrosine-rich store is in free solution. We do not know if it is in vesicles or not. The general impression is that the storage is very like that observed in the adrenal gland and, of course, very different from the store of insulin in the pancreatic vesicles. However, it is very similar to the state of the tyrosine-rich protein that is free in the haemolymph of insect larvae (Egmond *et al.* 1979), as seen by proton n.m.r. in whole 'animal' studies.

Seeds (with R. G. Ratcliffe and B. C. Loughman)

A very different situation exists within seeds (which store phosphate and fats) from that in the adrenal gland, since the seed is a germinating organ whence we can follow *changes* in the pool of ^{13}C, ^{1}H or ^{31}P. Study of dry seeds, prior to germination, by means of ^{13}C or ^{1}H n.m.r. revealed, as stated above, only the mobile fats. No protein is seen. ^{31}P n.m.r. at this stage shows only a broad spectrum which could be a composite of many lines near to inorganic phosphate or monoester phosphate. The signal is too ill defined to be easily attributed to any chemical species, but analytical knowledge of seed composition suggests that the most likely compound is a phytic acid. The chemical compound is not very mobile, as shown by the n.m.r. line shape. Soaking the seeds in D_2O causes swelling, but the seeds do not really germinate, even after long periods. Even so, there is a marked change in the ^{31}P signal, indicating that some phosphate is now in a freely mobile form, probably inorganic phosphate. (Note that we could follow uptake of water (D_2O) by studying n.m.r. relaxation times.) Soaking in H_2O allows full germination, of course, and new signals appear both in the ^{31}P and ^{1}H n.m.r. At a later stage, the ^{31}P spectrum of the evolving root and stem shows the presence of inorganic phosphate but no other unidentified phosphates or AMP, ADP or ATP. It is therefore inorganic phosphate, not a phosphate ester, which is transported. The proton n.m.r. spectrum of the stem reveals a wealth of compounds, including citrate. (Examination of the stems of a variety of plants has been carried out by means of ^{1}H n.m.r. spectroscopy, and we have observed a striking variety of compounds in the stems. There is much species selectivity. The compounds include some aromatics, probably phenols.) During the examination of many tens of seeds during germination we have not seen any signals from proteins. Yet extraction of soya beans produces at least one protein that is at least 50% in a highly mobile state (Egmond & Williams 1978). This observation is in striking contrast with those on the adrenal

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gland. We do not know the functional significance of the highly mobile portion of lipoxygenase of seeds and we suspect that it is a fault in method that has not allowed us to see this protein in the seed, as it is quite concentrated.

A very similar experiment can be performed with growing young plants rather than roots and stems evolving from seeds. An interesting experiment, as an example, is the study of phosphate-deficient oats. Using a 90 MHz (proton) spectrometer we could not really detect phosphorus in the stem of a plant which had been grown in phosphate-deficient media. When growth is on low phosphate, even 10^{-4} M, the signal due to inorganic phosphate could be seen in the shoot. The experiment shows clearly that it is *inorganic* phosphate that is transported *in vivo*.

The conclusion from several series of experiments on storage is quite simple. In *intact* storage vessels we now have clear n.m.r. evidence for different modes of storage. There is a somewhat viscous fluid of 'free' molecules in some glands, while in other storage units the components are virtually in crystals (cf. insulin). As the mobility data have accumulated, we have begun to collect a list of unusually mobile proteins. Already we have discovered that there are several types of such storage proteins and that these proteins do not have a tertiary fold. In some cases the proteins bind by covalent bonds to the compound, e.g. phosphate, to be stored, but in others, e.g. chromagranin, the function is more obscure. Proteins can act to adjust viscosity, to cross-link, or just to alter Donnan equilibria.

Locating organic molecules in space (with R. G. Ratcliffe and M. J. Kime)

The problem of discovering the location of *organic* chemicals in biological compartments, rather than just discovering their overall concentration in a tissue, is of obvious importance. For example, we wish to know which organic compounds are inside mitochondria rather than in the cell cytoplasm. Two approaches can be imagined. In the first a real map of the component is obtained by physical methods. In the second method, chemical kinetics are used to show the time sequence with which groups can exchange with others and it is presumed that the fast exchange occurs within a given compartment. The second method is not a true plot of biological space, but only a description of cocompartmentalization. An example is provided by the two adrenalin pools in figure 2.

Returning to the physical methods, we have attempted to use paramagnetic probes to locate the whereabouts of chemicals in space (table 2). The simplest experiment proceeds as follows. Let us suppose that a given chemical, X, is in two compartments, A and B, and that an added chemical can perturb the signal of X. If we find in which compartment (A or B) the perturbation has occurred, then we can locate separate signals due to X_A and X_B. As stated in the introduction, there are five basic parameters in the n.m.r. experiment: the energy of the signal, its splitting, its intensity and its two relaxation rates. We have to be able to locate in space the differentiated signals of X_A and X_B, and they can be differentiated only on the basis of shift (energy) and/or relaxation rates. To study the distribution of atoms in molecules (conformational analysis) we have previously used shift or relaxation probes, which are just ways of altering the effective field or the effective field fluctuation rate at a nucleus. There are simple equations governing the relation between the perturbation observed and the position in space of the observed nucleus, relative to the perturbing probe (see Levine & Williams 1975). The same principles apply to macroscopic plotting. Experimentally, we imposed a

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different field or a different relaxation rate in different parts of space. In the first instance, we can use the same probes as for microscopic studies. Thus, in a biological object, we alter the relaxation rates of certain signals when Mn II ions are added (Daniels *et al.* 1978; Lauterbur *et al.* 1979) and we can shift lines by adding Co II ions (Granot & Rosenheck 1979). Only those organic chemicals that have their n.m.r. signals perturbed are close to the perturbing metal ions. Thus, we achieve a map of chemicals in compartments, but not a map of the compartments in space. But, if we now combined these observations with the use of the electron microscope, then we can plot space. The analytical electron microscope will plot inorganic elements, giving their compartments, although it will not discriminate in space between organic compounds or even different phosphorus compounds. Therefore, if we observe perturbations of n.m.r. signals by probes into separated signals from different compartments and observe the

TABLE 2. MOLECULAR N.M.R. PERTURBATION PROBES

system studied	probe	reference
chromaffin granules, isolated and in the intact medulla	relaxation: Mn^{2+}, Gd^{3+} shift: Co^{2+}	Daniels *et al.* (1978)
red blood cell	susceptibility: lanthanide complexes	Campbell & Brown (1980)
plant vacuoles	various transition metal salts	

TABLE 3. MACROSCOPIC N.M.R. PERTURBATION PROBES

method	information	materials
straight wire of given susceptibility	resonance in proximity of wire broadened: variable diameter wire	Mo, W, coated Cu, Pt
variety of currents in wires	broadening of lines related to current and distance from the wire	coated Cu, Pt
coil of wire around material	resonances shifted if atoms inside coil	coated Cu, Pt
thin tubing containing paragmagnetic solutions	susceptibility changes	Fe III, Mn II solutions

compartment containing the probe by analytical electron microscopy, we have a plot in real space of the origins of the n.m.r. signals. The resolution of electron microscopy far exceeds anything we can hope for by direct n.m.r. methods of spatial discrimination. Unfortunately, the electron microscope cannot be used *in vivo*, but, of course, once a biological system has been calibrated, the n.m.r. studies can be used on their own. The method has been tested on model systems (Skarnulis *et al.* 1978).

A quite different approach to the definition of space is to adjust the field of the n.m.r. experiment (table 3). The method, which also forms the basis of the experimental attack presented in the second part of this symposium, is the use of a small field gradient imposed upon the large external field. Our method is to adjust the field *locally* by using macroscopic insertions. Experiments along these lines have been developed by Dr C. M. Dobson, Dr R. G. Ratcliffe, Mr M. J. Kime and myself. It is required to produce a very local field or local susceptibility that will change the resonance frequency. The simplest local perturbation can be obtained by introducing a fine wire, with or without a current flow, into the sample. As in zeugmatography, the first experiments were conducted on solutions in coaxial tubes (figure 6).

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In the narrow, inserted central tube A we have a wire or a surrounding coaxial coil. In figure 6 we illustrate a mock-up ^{31}P n.m.r. experiment in which three coaxial tubes are used. The line shapes in the top figure are grossly affected by these coaxial glass tubes and are further modified by the wire. (These are susceptibility effects and must be taken into account in all *in vivo* studies (see above).) The susceptibility is very dependent on the choice of the containing materials,

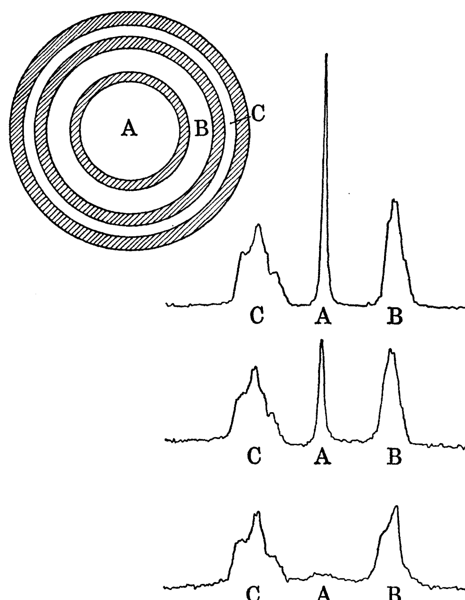

FIGURE 6. Top left, the model system comprising Wilmad 5, 8 and 10 mm diameter tubes, arranged coaxially, containing: A, *ca.* 200 mM inorganic phosphate in H_2O at pH 3.02; B, *ca.* 260 mM creatine phosphate in H_2O at pH 7.74; C, *ca.* 700 mM inorganic phosphate in D_2O at pH 9.67. A wire of diameter 0.25 mm runs centrally down tube A and returns as a loose helical coil. The ^{31}P spectra show the signals from the three compartments, and the broadening of resonances B and C, caused by the magnetic susceptibility differences in the system, is clearly visible. Spectra from the stationary system were recorded with the following currents: top, no current; middle, 1.3 mA; bottom, 29 mA.

glass, plastic, bone, skin, etc., and on the choice of metal for the wire. Electric currents of slowly increasing strength were now applied to the wire and the line half widths observed (figure 6). Linear plots of line widths against current for the three components in A, B and C showed that the line broadenings of the three were proportional to the distance of the components from the wire. Thus we have a one-dimensional plot of space. In a real experiment the wire (or several wires) can be located in different parts of space whence one-dimensional plots are obtained from several origins. It is clearly possible, by altering the geometry of the wire(s), to make a multitude of probe devices; coils can be used in place of linear wires, for example. There is no need to invade a biological object with these wires, although our first successful tests on plant stems have used such an insert. Obviously it is also possible to adjust the field locally by employing *external* field gradients (see later papers in this symposium report).

The future use of high resolution n.m.r. will depend greatly on increased sensitivity and on the ability to probe space. Even now, however, we can see that n.m.r. can establish confidence in the relation between *in vitro* and *in vivo* conditions (Torchia *et al.* 1977).

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