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WHOLE ORGAN METABOLISM STUDIED BY NMR

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INTRODUCTION

Nuclear magnetic resonance (NMR) is now established as a noninvasive method of studying metabolism in vivo, and in this review we discuss the contribution that this technique can make to our understanding of cellular metabolism. Since 1979, in vivo NMR has been the subject of a large number of reviews (18, 39–41, 44, 46, 55, 56, 63, 88, 90, 96, 97, 99), and in order to avoid excessive overlap this review concentrates on the most recent developments that have taken place. There have been some notable advances during the last two or three years, and the most eye-catching probably has been the application of NMR to human metabolism.

The emergence of a new technique for studying metabolism inevitably invites comparison with the more traditional methods that are available. The main feature of NMR is that it is nondestructive and noninvasive, and so metabolic processes can be followed as they take place within a living system. In addition, certain aspects of the intracellular environment can be monitored; for example, the intracellular pH is commonly measured by ^{31}P NMR using the frequency dependence of the inorganic phosphate signal. Furthermore, it is possible to study reactions taking place in vivo under steady state or equilibrium conditions. The main disadvantage of the technique is that it is insensitive, and therefore it can directly detect only those metabolites that are present at concentrations of about 0.2–0.5 mM or above. For this and a variety of other reasons, NMR should be regarded as a technique that is complementary to, rather than competing with, the other methods that are available for studying metabolism.

The majority of metabolic NMR studies have focused on the ^{31}P nucleus. Spectra can be obtained reasonably rapidly (typically in about a minute), they can be interpreted very readily, and they are highly informative; often

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only five or six signals are observed, but they are from phosphorylated metabolites such as ATP that give an excellent reflection of the intracellular energy status. In addition, ^{31}P NMR spectra can provide a continuous monitor of the intracellular pH.

The other nuclei used for metabolic studies are ^{1}H, ^{13}C, and to a lesser extent ^{15}N. All of these nuclei have characteristic advantages and drawbacks. The abundant isotope of carbon, ^{12}C, has no magnetic properties and does not produce NMR signals. Therefore ^{13}C, which is only 1% abundant, is used. In order to obtain detectable signals, it is usually necessary to enrich the sample with ^{13}C-labelled material, and in this way it is possible to perform ^{13}C NMR studies that are analogous in many respects to radioactive tracer experiments using ^{14}C. Unfortunately, a large amount of ^{13}C-label must be used, and this may be very expensive.

^{15}N can be used in a similar manner to ^{13}C; the advantage is that in contrast to carbon, there is no suitable nitrogen isotope that can be used for radioactive tracer studies. The disadvantage is the very low sensitivity of ^{15}N; as yet there have been only a few reports of the use of ^{15}N NMR in metabolic studies (e.g. 95).

^{1}H NMR is much more sensitive than ^{31}P or ^{13}C NMR, and therefore in favorable circumstances it can detect metabolites that are present at concentrations below 0.2 mM. In addition, because of the ubiquity of the ^{1}H atom, a very large number of compounds are in principle accessible to study. However, this ubiquity of ^{1}H atoms also leads to problems, first because the spectra contain so many overlapping signals that they defy interpretation, but also because water produces an enormous signal that can mask the resonances of interest. These problems have been overcome for studies of cellular suspensions (15, 16, 85, 100, 101), but it remains to be seen whether they can be overcome for studies of whole organs.

Although the large ^{1}H signal from water is a nuisance in studies of metabolism, it can be used to great advantage for NMR spin imaging. This is a technique that provides information about the spatial distribution of water throughout a sample. Different tissues can be distinguished from each other on the basis of their differing water contents and NMR properties, and excellent images have been obtained of the human head and whole body (e.g. 108). NMR spin imaging is likely to have a major impact in medicine but is discussed in this article only where it relates to metabolic studies. ^{1}H NMR can also be used to measure flow (11, 78, 89), and there is much interest in the possibility of combining metabolic studies using ^{31}P NMR with the measurement of blood flow by ^{1}H NMR. Another nucleus that is suitable for imaging studies is ^{23}Na (32), and it could also prove to be of value for studies of flow and of transport (59).

Many of the advances in this rapidly expanding field of research have

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relied on recent technological developments, and we therefore begin the review with a discussion of the technology of in vivo NMR, stressing in particular the differences between this branch of NMR and the more conventional NMR studies of solutions. We then discuss the applications of NMR to whole organs, concentrating (for the reasons mentioned above) on ^{31}P NMR. Studies of cellular suspensions are discussed only where they are relevant to the whole organ studies.

THE TECHNOLOGY OF IN VIVO NMR

The basic principles that govern metabolic NMR studies are no different from those involved in more conventional NMR experiments on solutions. However, there are a number of practical differences that not only affect the way in which experiments are set up and performed but that also influence the choice of spectrometer to be used. For this reason, it is useful to be aware of the technology that is special to in vivo NMR. Further details can be found in reference (40).

Probe Design

For conventional NMR studies of solutions, excellent spectral resolution is required (better than 1 part in 10^8), and so the design of the probe, which contains the sample and radiofrequency coil, requires a great deal of skill and precision. In contrast, field homogeneity of about 1 part in 10^7 is usually sufficient for metabolic studies, and therefore the constraints on probe design are not so great. This means that there is more scope for versatility in probe design. In particular, (*a*) there is no need to spin the sample; (*b*) the sample chamber and radiofrequency coil can be designed to suit the sample under investigation; and (*c*) it is possible to incorporate perfusion techniques, together with devices for monitoring physiological function within the spectrometer, without prejudicing the quality of the NMR itself.

Probes have been specifically designed for studies of isolated skeletal muscle (28), perfused hearts (47, 72), perfused livers (65), and intact animals (40). Sample chambers designed for cellular suspensions (10, 48) and tissue culture (105, 107) have also been described.

Locking and Shimming the Magnetic Field

Before any experiment, it is necessary to "shim" the field, conventionally using a ^2D signal from deuterium oxide that is added to the sample. This can be a problem in studies of living systems, as the addition of D_2O to the sample itself is usually unacceptable, and it may be impractical to introduce a capillary of D_2O into the sample chamber. However, it is generally

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possible to shim the field using, instead of a ^2D signal, the ^1H signal from water; the method described by Ackerman et al (2) is thoroughly recommended for its convenience and ease of use. A modification to this method which leads to a larger ^1H signal has been described by Gordon & Timms (52).

The ^2D signal from D_2O is also routinely used in conventional experiments for the field-frequency lock. However, because of the stability of modern superconducting magnets, and because the requirements for spectral resolution are not so stringent for in vivo studies, a lock is not generally required for studies of this type. Therefore D_2O is not needed.

The Use of Surface Coils

The radiofrequency coils that are used for conventional NMR studies are not suitable for most studies of whole animals, partly because of the need to localize on a specific region of the animal. Metabolic studies of selected tissues and organs within whole animals became feasible through the introduction of surface coils (3). A surface coil in its simplest form consists merely of a flat circular loop of wire. If such a coil is placed adjacent to an object, under normal circumstances it will detect signal from an approximately disk-shaped region of the sample immediately in front of the coil, of radius and thickness similar to the radius of the coil. A surface coil therefore provides a convenient and remarkably simple method of "localizing" on a region that is close to the surface of the sample, and it is ideally suited for examining the metabolic state of muscle and brain in vivo. Surface coils have also been used to investigate regional metabolism within isolated perfused hearts (83).

One of the difficulties associated with surface coils is that their radiofrequency field is inhomogeneous. This can lead to problems when measuring relaxation times, but the problems can be reduced by the use of "composite" radiofrequency pulses (38). For example, such pulses have been used for T_1 measurements in ^{31}P NMR studies of the rat brain (98).

Topical Magnetic Resonance

Surface coils can be used to study internal organs such as the kidney or liver, if coupled with surgery (14, 57). However, there are obvious disadvantages of such an approach. One technique successfully used for "localizing" on internal organs has been termed topical magnetic resonance (50, 51, 61). This technique employs special field homogeneity coils, which profile the magnetic field in such a way that the field is very homogeneous over a central, approximately spherical volume but elsewhere is very inhomogeneous. As a result, high resolution signals are observed only from the central region. Studies have been performed on the liver of intact rats (51)

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and also, by combining topical magnetic resonance with the use of surface coils (9), on the kidney.

The Magnet

There are problems associated with the cost and, in extreme cases, the technical feasibility of building wide-bore magnets of high field strength. For this and additional reasons, a suitable field strength for ^{31}P and ^{13}C studies of human metabolism is about 2 T, but it is only recently that such magnets have become available. Higher fields (about 4–9 T) are generally used for studies of isolated organs and small animals, but it is not necessarily advantageous to use the highest available field (40). This contrasts with solution studies, for which the highest available field is generally desirable.

Imaging and Metabolism

There is much interest in the possibility of combining a spin-imaging technique with metabolic studies in order to image the metabolic state throughout a given region (e.g. 12). Unfortunately, there are severe sensitivity problems associated with spin imaging of ^{13}C- or ^{31}P-containing metabolites, primarily because they are present in vivo at relatively low concentrations, and therefore the attainable spatial resolution would be extremely poor. For this and additional technical reasons, the emphasis in metabolic studies of whole animals and humans has been to "localize" on specific regions, using surface coils, topical magnetic resonance, or a mixture of the two approaches, as outlined above.

WHAT CAN NMR TELL US ABOUT METABOLISM?

In this section, we discuss in general terms the type of information that is available from in vivo NMR studies. In subsequent sections, we illustrate the scope of these studies by describing some selected applications in more detail.

Metabolite Levels

High resolution NMR signals are generally observed only from mobile compounds, because highly immobilized compounds give rise to signals that are often too broad to observe. Therefore, an important distinction between NMR and freeze clamping measurements of metabolite levels is that NMR detects only the mobile components, whereas freeze clamping detects the total amounts of metabolites.

Provided that the appropriate controls are performed (in particular for the effects of signal saturation), the areas of NMR signals are proportional

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to the concentrations of the metabolites that give rise to them. Therefore, relative concentrations of metabolites can be determined fairly easily. The measurement of absolute concentrations is more difficult, and it requires the use of special calibration procedures (28, 47). Absolute quantitation when using surface coils is complicated by the fact that different regions of the sample generate different amounts of signal, depending on their location relative to the coil. Therefore, surface coil studies have so far been restricted to measurements of relative concentrations. However, this usually provides a sufficient basis for interpretation. Moreover, absolute concentrations can often be deduced by comparing the relative concentrations as measured by NMR with the total metabolite levels determined from freeze-clamping studies.

It should be noted that concentration measurements are not restricted to those compounds that generate detectable signals. For example, there are methods for deducing the levels of free Mg^{2+}, ADP, AMP, and $NAD^+ + NADH$, as described below.

NMR provides an excellent method of examining the poorly understood relationships between tissue function and metabolism; changes in metabolic state can be followed continuously and can be related to simultaneous measurements of function. Typically, the time required to accumulate a spectrum is about 1 min, which limits the time resolution for this type of study. However, when cyclical changes occur, the time resolution can be greatly enhanced by synchronizing the collection of NMR data with different parts of the cycle (28, 37, 69).

The Intracellular Environment

NMR is now established as an important noninvasive method for monitoring intracellular pH. Most measurements make use of the inorganic phosphate signal, the frequency of which is sensitive to pH variations in the normal physiological range. In general, however, any NMR signal that responds to pH changes can provide a monitor of intracellular pH, and several other compounds have been used, including 2,3-diphosphoglycerate (75), ATP (see 41), deoxyglucose 6-phosphate (8), and methyl phosphonate (48). In addition, the ^1H NMR signals from the histidine resonances of hemoglobin have been used to measure the pH of red blood cells (16). The observation of multiple pH environments can sometimes be used to study compartmentation (see 41, 90).

NMR signals can also provide information about metal ion binding, as described below.

Reactions in the Steady State

NMR can be used to measure the rates of reactions taking place under steady state or equilibrium conditions. One approach, used in ^1H and ^{13}C

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studies, involves the use of isotope exchange and incorporation. A second approach uses the technique of saturation transfer NMR (36), which is a type of magnetic labelling experiment. If the magnetization of one species is perturbed, this perturbation can be transferred to a second species if the two are in exchange with each other. The extent to which the second species is affected (together with a measurement of the spin-lattice relaxation time T_1) gives a measure of the rate of interconversion.

Apart from the intrinsic value of measuring reaction rates in vivo, it is of considerable interest to compare enzyme activities in vivo and in vitro in order to examine the extent to which activity is modified by the intracellular environment. The first results are just beginning to emerge.

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METABOLITE LEVELS IN VIVO

As mentioned above, high resolution NMR generally detects only mobile compounds. For frog sartorius muscle, freeze clamping and NMR provide similar values for the concentrations of ATP, phosphocreatine, and inorganic phosphate (28), which indicates that only a small fraction of these compounds can be substantially immobilized. In contrast, numerous studies on various tissues show that NMR measurements of ADP and inorganic phosphate are much lower than those measured by freeze clamping.

The direct measurement of ADP levels by NMR is a problem because its two signals overlap with two of the three signals from ATP; this makes it difficult to quantify ADP if its contribution to the signal intensities is very much lower than that of ATP. However the lack of detectable signal from ADP is itself of interest, for this means that one can place an upper limit on the concentration of free ADP. Thus it can be concluded that in the brain (3) and kidney (9) of anesthetized rats, the concentration of free ADP must be very much lower than the total values measured by other means. Furthermore, even if the ADP cannot be directly measured, the concentration of free ADP can sometimes be evaluated if the creatine kinase reaction is at equilibrium. This reaction has been shown by saturation transfer NMR (see below) to be at equilibrium in resting frog gastrocnemius muscle (42), in cat biceps muscle (74), and in the perfused rat heart (70). The concentration of free ADP can be calculated to be about 20 μM in the two types of skeletal muscle (42, 74) and somewhat higher in the perfused heart (73), the precise value in the heart depending on the substrate that is used. These concentrations of free ADP are very much lower than the values for total ADP that have been measured by other methods, but are in good agreement with the conclusions of Veech et al (106). By making use of several reactions that are believed to be close to equilibrium, they calculated

that the concentrations of free ADP in brain, muscle, and liver were 32, 37, and 46 μmol/l cell H_2O respectively.

Several factors could account for the difference between the NMR and freeze-clamping measurements. For example, in muscle it is known that a large percentage of the ADP is tightly bound to the proteins of the myofilaments. In addition, for many tissues there may be an unavoidable breakdown of high-energy phosphates during the process of freeze clamping and subsequent extraction. This could also explain the low inorganic phosphate levels measured by NMR. A further possibility is that a significant fraction of the intracellular ADP is sequestered, e.g. in the mitochondria, in such a way that it generates no detectable signal. Recent NMR studies of isolated mitochondria indicate that they can contain pools of ADP and ATP that are "invisible" to NMR (109).

The concentration of inorganic phosphate measured by NMR is in the range of 1–2 mM in the muscle (3) and brain (98) of anaesthetized rats, and numerous NMR spectra suggest similar values in other tissues. Again, these NMR values are lower than many reported measurements of inorganic phosphate concentrations [see, for example, data in (106)]. As is the case for free ADP, the concentration of inorganic phosphate in the perfused heart depends on the substrate that is used (73). Inorganic phosphate in the rat heart can also be affected by treatment with adrenaline (73) or insulin (6). During normoxic perfusion with buffer containing 5 mM glucose, insulin increases the ratio of phosphocreatine to ATP while reducing the intracellular inorganic phosphate to a level that is barely detectable. However, no change is observed when acetate is used as the sole substrate. This effect must therefore be dependent upon the effect of insulin on glucose transport and glycolysis.

The low concentrations of ADP and inorganic phosphate must affect our views about the control and thermodynamics of cellular metabolism. For example, there is still much controversy regarding the mechanisms whereby respiratory control is achieved. Jacobus et al (68) have recently suggested that the most plausible explanation of respiratory control is the availability of ADP and the kinetics of its transport by the adenine nucleotide translocase. This hypothesis, which was first proposed by Chance & Williams almost 30 years ago (22), is entirely consistent with the low concentrations of free ADP. Matthews et al (73), on the basis of their studies of perfused rat hearts, concluded that there is no general correlation between the phosphate potential and mitochondrial respiration. However, this does not necessarily conflict with the suggestion of Jacobus et al, as additional factors could also affect the rate of mitochondrial respiration. The control of glycolysis and glycogen metabolism must also be reconsidered, particularly if, as seems likely, the concentration of free AMP is

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also very low (it can be calculated to be about 0.1 μM in resting muscle if the adenylate kinase reaction is at equilibrium). Griffiths (54) has suggested that the low level of inorganic phosphate may be a factor that limits the rate of glycogen breakdown in resting muscle.

The low concentrations of ADP and inorganic phosphate mean that the cytosolic phosphorylation potential and the free energy of ATP hydrolysis are greater than previously believed. This is of importance in our considerations not only of oxidative phosphorylation but also of a variety of active transport processes, such as the transport of Ca^{2+} into the sarcoplasmic reticulum (30).

The activity of lactate dehydrogenase in intact erythrocytes has been measured by ^{1}H NMR (15, 100, 101). It was concluded from these measurements that the free $NAD^+ + NADH$ concentration in the erythrocytes is only about 10% of the total $NAD^+ + NADH$ and that this low free concentration limits the activity of the lactate dehydrogenase within the cells. The majority of the intracellular $NAD^+ + NADH$ is presumably bound to various dehydrogenases and reductases. These results, together with the measurements of free ADP and inorganic phosphate, demonstrate the need to estimate free as well as total concentrations of metabolites in vivo and illustrate the importance of combining NMR measurements with measurements that are available from the more traditional methods for studying metabolism.

Changes in metabolic state can be followed during muscular contraction (28–31) and during conditions such as ischemia (1, 6–8, 14, 47, 69, 80, 93, 94) and shock (20, 87). Much of this work has been included in earlier reviews. In a relatively recent study, ^{31}P NMR spectra have been observed from the intact crystalline rabbit lens (53). The use of NMR in conjunction with functional and morphological analysis could possibly increase our understanding of cataract development.

THE MEASUREMENT OF INTRACELLULAR pH

The Reliability of pH Measurements

It is generally accepted that ^{31}P NMR can reliably measure intracellular pH; the values obtained by NMR in a variety of samples under a variety of conditions agree well with anticipated values (e.g. 28, 82, 94, 102). Nevertheless, it is essential to be aware of the possible pitfalls of the method. These have been discussed in detail elsewhere (40, 43, 44, 48, 67, 69, 90, 91) but are mentioned briefly below.

First, there is bound to be some uncertainty about the precise intracellular conditions, and therefore it is essential to ascertain whether factors other than pH might affect the chemical shift of the signal that is being used

for the pH measurements. Fortunately, control experiments (e.g. 44, 48, 67, 69, 91) have shown that the effects of likely variations in ionic strength or metal ion binding within living systems are small and can in general be ignored. Nevertheless, since large changes in these parameters can produce significant shifts (44, 48, 67, 91), it is important to ensure that the ionic composition of the standard calibration solution resembles as closely as possible the conditions expected in vivo. It is far more difficult to take into account the effects of binding to macromolecules. However, it has been found that inorganic phosphate titration curves are similar in simple aqueous solution and in homogenized heart preparations (69), which suggests that phosphate-protein interactions have little effect on the observed chemical shift of inorganic phosphate.

Second, it may be difficult to find a suitable reference compound. For ^{31}P NMR, phosphocreatine often provides a convenient internal standard, but when this is not present, the ^{1}H NMR signal from water can be used (2). Methylene diphosphonate can be used as an external standard for ^{31}P NMR, but there are problems associated with the use of phosphoric acid (40, 44).

Third, it is important to establish which intracellular compartment is being monitored. Experiments utilizing 2-deoxyglucose (8) indicate that in the rat heart the inorganic phosphate signal monitors cytoplasmic rather than mitochondrial pH, and it is likely that this is also true for other tissues.

Fourth, there may occasionally be some doubt as to the assignment of the signal that is to be used for pH measurements. For example, one of the signals from 2,3-diphosphoglycerate (2,3-DPG) has a frequency similar to the inorganic phosphate signal, and it has been suggested that 2,3-DPG may make a significant contribution to the ^{31}P spectrum of the rat brain (3). If this were the case, it would be difficult to measure the intracellular pH. However, it has been estimated that the effective concentration of 2,3-DPG is in fact too low to make a significant contribution to the observed brain spectra (98).

The main uncertainty is probably associated with the standard calibration curves, which are generally obtained by plotting the chemical shift of the required compound in solution against the solution pH measured with a combined glass electrode. Illingworth (66) has pointed out that such electrodes can produce pH measurements that are in error by as much as 0.2 pH units or more. This could account for the fact that different pH titration curves that have been reported in the literature differ slightly from each other. In view of the various possible uncertainties, it is unwise to hope for absolute accuracy of better than 0.1 pH unit when measuring intracellular pH by NMR, but changes in pH can often be measured to better than 0.05 pH unit.

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Some Applications of pH Measurements

^{31}P NMR has been extensively used to study the metabolic events associated with ischemia and recovery in a range of organs [see above; see also (72) for studies of cardiac metabolism]. As an example of these studies, it has been shown that in perfused rat hearts (47) and kidneys (14), the decrease in pH (in hearts from about 7.05 to 6.2) is considerably reduced by the presence of the buffer bis-tris-propane in the perfusion medium. This appears to have a significant protective effect on the ischemic tissue. In further studies, Jacobus et al (69) have studied the role of intracellular pH in the regulation of myocardial contractility. Their results demonstrate a fairly tight coupling between pH and contractility in the normal heart during respiratory acidosis. However, during total global ischemia, the extent of acidification cannot fully account for the observed contractile depression; other metabolic and/or physiological mechanisms must also contribute to the effect.

The decline in pH during ischemia (47) and during muscular fatigue (29) can be interpreted in terms of the anaerobic breakdown of glycogen to lactate and therefore provides a method of studying the rate of glycogenolysis. Interesting conclusions are emerging from these studies about the control of phosphorylase and phosphofructokinase in vivo. For example, the phosphorylation of 2-deoxyglucose in perfused rat hearts has been monitored by ^{31}P NMR (8). 2-deoxyglucose 6-phosphate inhibits phosphorylase b in vitro, and from the inhibitory effect of this compound on glycogenolysis during ischemia, it was estimated that after 5 min of ischemia the b form of phosphorylase makes a significant (about 50%) contribution to glycogen breakdown. In contrast, from NMR studies of frog (31) and human (34, 86, 102) skeletal muscle, it seems probable that in these systems Ca^{2+} ions are required for activation of the phosphorylase system.

Infusion of insulin into rat hearts prior to ischemia causes an increase in the rate and extent of acidosis during the period of no flow, while the rate of ATP depletion is decreased. This has been interpreted in terms of an increased content and accessibility of glycogen as a result of the insulin treatment (6). These studies show that glycolysis and hence phosphofructokinase activity are maintained in the ischemic heart, despite a decrease in intracellular pH to below 6.0. This enzyme is inhibited in vitro by a decline in pH, but it is important to bear in mind that a pH decline in vivo is often accompanied by additional metabolic changes that can also modify the activity of this enzyme.

In this respect, there has been an interesting suggestion (see 35) that insulin might increase intracellular pH and thereby regulate glycolysis

through the effect of pH on phosphofructokinase activity. In frog sartorius muscle, insulin does increase pH, as measured by the weak acid dimethadione (DMO) (76) or by ^{31}P NMR (77), and additional studies support this suggested action of insulin (35). However, ^{31}P NMR studies of perfused rat hearts provide no evidence for an increase in intracellular pH in the presence of insulin (6). This may reflect a greater capacity for pH regulation in the homeothermic mammalian cardiac muscle than in the poikilothermic amphibian skeletal muscle.

^{31}P NMR spectra have been observed from tumors (Walker 256 carcinomas) in the living rat (58). Contrary to expectation, the intracellular pH was only slightly more acid than in the normal rat muscle, and it did not fall significantly after infusion of glucose. It was suggested that the tumor may have a mechanism for expelling excess protons into the surrounding medium. A similar mechanism may exist in some people with mitochondrial disorders (86; and see below).

Finally, there is much interest in the role of the pH changes that are associated with the activation of metabolic processes in a wide range of organisms (see 81). ^{31}P NMR has been used to measure the intracellular pH of unfertilized, fertilized, and activated eggs of *Xenopus laevis* (82). pH values of 7.42, 7.66, and 7.64 respectively were obtained, and these values are almost identical to those obtained using pH-sensitive microelectrodes. This experiment provides supportive evidence for the reliability of these two methods of measuring intracellular pH.

MEASUREMENT OF INTRACELLULAR FREE Mg^{2+}

The frequencies of the ^{31}P signals of ATP are sensitive to the binding of divalent metal ions to ATP [(27); see also (44) for pH titrations of free ATP and MgATP performed at physiological concentrations]. It was concluded from the earliest whole tissue studies (64) that the ATP in skeletal muscle is predominantly complexed to Mg^{2+} ions, and similar conclusions regarding the state of ATP have been reached for other tissues. This result, together with measurements of free Mg^{2+}, is of importance when considering those reactions that require MgATP and MgADP as substrates, and also those enzymes for which Mg^{2+} binding is required for activity.

In principle, it is possible to calculate the concentration of free Mg^{2+} in vivo from the frequencies of the ATP signals, provided that the MgATP dissociation constant is known under intracellular conditions and also that no factors other than pH or Mg^{2+} binding affect the ATP frequencies. In this way, Gupta & Moore estimated that the concentration of free Mg^{2+} in frog skeletal muscle is 0.6 mM (60). However, Wu et al (110), using a different effective dissociation constant, obtained a value of 2.5 mM both in

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frog skeletal muscle and in guinea pig hearts. This value remained unchanged during ischemic arrest of the hearts.

Cohen & Burt (23) used a different ^{31}P NMR approach, based on measurements of the phosphocreatine relaxation times, to obtain a value for free Mg^{2+} of 3.0 mM in frog muscle. Although these NMR methods are subject to fairly large errors and rely on assumptions that have yet to be verified (see 41), the results are in gratifying agreement with those recently obtained by Hess & Weingart (62) using Mg^{2+}-selective microelectrodes. They found that concentration of intracellular free Mg^{2+} was 3.5 mM in sheep Purkinje fibers, 3.1 mM in sheep and ferret ventricle muscle, and 3.3 mM in frog skeletal muscle. Metabolic poisoning of the Purkinje fibers with 0.1 mM 2,4-dinitrophenol did not produce changes in the Mg^{2+} level.

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REACTIONS IN THE STEADY STATE

^{13}C and ^{1}H Studies

There have been many ^{13}C metabolic studies of cellular suspensions, but relatively few of intact organs. Following earlier studies of hepatocytes (24), Cohen et al have obtained ^{13}C NMR spectra from mouse livers perfused with ^{13}C-labelled alanine and ethanol (26). Signals are observed from individual carbons within various metabolites, and their intensities and spin-coupling patterns can provide detailed information about the relative rates of many of the reactions of gluconeogenesis. For example, the distribution of label in glucose shows that there is significant pentose cycle activity, and also that the triose phosphate isomerase reaction is close to equilibrium.

In further studies of liver metabolism, ^{13}C NMR has been compared with ^{14}C tracer studies in order to evaluate the reliability of the ^{13}C NMR method (25). ^{13}C NMR was used to monitor the gluconeogenic flux from glycerol, the synthesis of glycogen, the stimulation of glycogenolysis by glucagon, the recycling of triacylglycerol, and an increase in pentose cycle activity under the influence of phenazine methosulphate. The relative concentrations of ^{13}C label at specific carbons as measured by NMR agreed closely with ^{14}C measurements on extracts of the same doubly labelled samples for specific activities of about 3% and greater.

Bailey et al (5) have investigated the incorporation of ^{13}C into amino acids in isolated rat hearts perfused with sodium [2-^{13}C] acetate. Their studies of extracts and of the whole tissue suggest that the method could be of general use in metabolic studies of the perfused heart.

^{13}C NMR has also been used to follow the conversion of [1-^{13}C] glucose to liver glycogen in the rat in vivo. A ^{13}C signal from glycogen appeared after 75 min but subsequently decreased in intensity, presumably due to

mobilization of glycogen (4). Signals from ^{13}C in natural abundance have been observed from the head, liver, and hind leg (4) of the rat and from the human forearm (4, 34, 49). The signals can be assigned to groups within triglycerides and lipids, which are present at sufficiently high concentrations to be detectable in the absence of enrichment. The relative intensities of the various signals could be of value in characterizing nutritional fat deficiency and abnormalities in the fatty acid pathways. ^{13}C signals have similarly been observed from intact isolated chicken muscle (33).

In ^{1}H NMR studies of intact erythrocytes, it has been possible to measure the activity of lactate dehydrogenase within the cells by observing isotope exchange (^{1}H-^{2}D) between lactate and pyruvate (15, 100, 101). The inhibition of the enzyme in intact cells by both oxalate and pyruvate was found to be similar to that of the purified enzyme, and it was concluded that the activity of lactate dehydrogenase within the erythrocytes is limited by the low concentration (about 10 μM) of free $NAD^+ + NADH$. It remains to be seen whether isotope-exchange studies of this type can be extended to intact organs.

Saturation Transfer Studies

As mentioned above, the technique of saturation transfer NMR can be used to measure reaction rates in vivo under steady state conditions. The method has been most widely applied using ^{31}P NMR to study the activities of creatine kinase and ATP synthetase.

In anaerobic resting frog muscle at 4°C, it has been shown that the creatine kinase reaction is close to equilibrium (42). However, this is not true during contraction when, contrary to expectation, the creatine kinase reaction is not considerably faster than the rate at which ATP is utilized. Nevertheless, the measured rates do account for the well-known observation that during contraction the ATP concentration falls by less than 2–3%. It was suggested that during exercise, the formation of a nonproductive enzyme-creatine-ADP complex could play an important regulatory role. The creatine kinase reaction is also close to equilibrium in resting cat biceps muscle (74). The flux measured in this preparation was similar to that measured in model solutions containing the same specific activity of enzyme, which suggests that the activity of creatine kinase is not modified by its localization within the muscle cells.

Following earlier studies of creatine kinase in the perfused rat heart (1, 17, 84), the activities of both creatine kinase and ATP synthetase have now been measured (70). It was concluded that in the Langendorff-perfused heart using glucose as the substrate, the rate of ATP synthesis is over five times slower than the unidirectional flux through creatine kinase. Thus the

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creatine kinase reaction is close to equilibrium. From measurements of oxygen consumption, the apparent mitochondrial ADP:O ratio was estimated to be 3.5 ± 0.8, which is not significantly different from the commonly accepted value of 3, first proposed by Ochoa 40 years ago.

The activity of creatine kinase has also been measured in the Langendorff-perfused rat heart utilizing oxygen at different rates and using either glucose or acetate as substrate (71). Under all studied conditions, it was found that the flux through creatine kinase was considerably greater than the rate of ATP utilization, again confirming that the creatine kinase reaction is close to equilibrium. It was concluded that the cytosolic free ADP level was primarily responsible for regulating the creatine kinase flux; the enzyme displayed a K_m for cytosolic ADP of 35 μM, a value in reasonable agreement with in vitro measurements using the isolated enzyme. In addition, the apparent V_{max} in vivo is 5.5 mM/s, which is only slightly smaller than the maximum extractable activity of the soluble form of the enzyme.

Similar studies of creatine kinase and ATP synthetase support the contention that the creatine kinase reaction is close to equilibrium in the rat brain (98). Thus the rather surprising observation that in the ischemic brain the levels of phosphocreatine and ATP fall in concert cannot be explained by low creatine kinase activity. It seems more likely that this effect reflects tissue heterogeneity with respect to energy demands. The measured activity of ATP synthetase in vivo agreed well with values predicted on the basis of oxygen consumption measurements.

In almost all of these systems, there is a discrepancy between the measured values of the forward and reverse fluxes through creatine kinase, despite the fact that the reaction is in a steady state. This apparent anomaly could in principle be explained in terms of the reactions that compete for ATP (e.g. ATP hydrolysis and synthesis or adenylate kinase activity) or alternatively in terms of compartmentation of the metabolites involved in the creatine kinase reaction. Possibly the use of the inversion transfer NMR method (this is closely related to saturation transfer) will establish which, if any, of these explanations is correct (74).

NMR Measurements of Oxygen Consumption

It has been shown that at fairly high magnetic field strengths, the oxygenation state of hemoglobin in blood can be determined from measurements of the spin-spin relaxation time T_2 of water protons in the blood (104). By combining these measurements with NMR measurements of blood flow, it is possible to monitor the oxygen consumption of tissues and organs in vivo. Unfortunately, the dependence of T_2 on field strength is such that measurements will not be feasible at the low field strengths used

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for clinical ^{1}H NMR imaging. However, this method of monitoring oxygen consumption could be of considerable value for animal studies, particularly when used in conjunction with high resolution ^{31}P NMR measurements of metabolic state. Preliminary studies of this type have been described (103).

HUMAN METABOLISM

With the development of wide-bore, high field (1.9 T) magnets, studies of human metabolism are now feasible. At the time of writing, the bore size (20 cm diameter) of available magnets permits the examination of isolated human organs and of human limbs, but by 1983 similar magnets, but of wider bore, should be available for whole body metabolic studies.

^{31}P NMR spectra containing signals from phosphocreatine (PCr), ATP, and inorganic phosphate (Pi) can readily be obtained from human forearm muscle (13, 21, 34, 87, 102). In their studies of control subjects, Taylor et al (102) have found that the intracellular pH of the finger flexor muscle is 7.04 $\pm$0.03 (s.d. $n = 20$) and the concentration ratios PCr : ATP and Pi : ATP are 4.5 and 0.46 respectively. Absolute concentrations can be evaluated from these ratios using the assumption (from biopsy studies) that the concentration of ATP is 5.5 mmol/kg wet wt. Slightly different ratios have been reported by Edwards et al (34). During aerobic exercise, phosphocreatine and pH both decline, and it is found that there is a fairly good correlation between intracellular pH and the ratio PCr : PCr + Pi (102). The recovery of phosphocreatine following exercise is normally half completed in about a minute (102).

These observations agree well with previous biopsy studies with the exception that, perhaps as anticipated, the ratio of phosphocreatine to inorganic phosphate is much higher when measured by NMR than by biopsy. In addition, the two types of study differ in the extent of the pH decline that is measured during exercise. ^{31}P NMR shows that the intracellular pH falls to as low as 6.0; one subject could exercise for more than 10 min with an intracellular pH of between 5.9 and 6.1 (102). In contrast, biopsy studies have shown that after exercise to exhaustion, the intracellular pH in the quadriceps muscle falls to about 6.4 to 6.5. This difference may reflect the different muscles that are being investigated. The NMR results suggest that glycolysis, and therefore phosphofructokinase, must be at least partially active at pH 6.0, in agreement with the studies of insulin-treated hearts (6) that are described above.

It was found that during a 6 min ischemic period following exercise, there is no detectable metabolic recovery (102). This is interesting first because it means that glycolysis is inactivated at the end of exercise, despite elevated levels of AMP and ADP, but second because metabolic recovery must

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therefore be almost exclusively oxidative. Thus the time course of metabolic recovery provides information about the capacity of oxidative metabolism. This lack of glycolytic activity after exercise is consistent with the additional finding that glycolysis is very slow in human forearm muscle when exposed to a prolonged period of ischemia in the absence of exercise (34), and suggests that Ca^{2+} ions are required for activation of the phosphorylase system. A similar conclusion was reached from NMR studies of frog gastrocnemius muscles at 4°C (31), as mentioned above.

These NMR studies provide a fresh insight into several different aspects of muscle biochemistry and physiology, but they also give essential baseline information for studies of diseased states. Significant deviations from normality have been observed by ^{31}P NMR in several patients with established clinical disorders. For example, in patients with McArdle's syndrome (a defect in glycogen metabolism), the muscle pH becomes alkaline during exercise, rather than acid (92), which is totally consistent with the inability of these patients to generate lactic acid from glycogen. A patient with phosphofructokinase deficiency not only became alkaline on exercise but also accumulated sugar phosphate, again as anticipated (34). ^{31}P NMR spectra of a boy with myopathy, ophthalmoplegia, and raised oxygen consumption showed an abnormally high ratio of inorganic phosphate to phosphocreatine at rest (45). This finding was consistent with the abnormality of mitochondrial function already established by biopsy, and the raised oxygen consumption in the patient could be explained by elevated levels of inorganic phosphate and ADP. In two sisters with a mitochondrial disorder (86), recovery of phosphocreatine following exercise was very slow, demonstrating a reduced rate of oxidative metabolism. However, pH recovery was faster than in controls. Moreover, in view of the high blood lactate levels that had been observed in one of the patients, the changes in intracellular pH on exercise were rather milder than might have been expected. It therefore seems possible that such people have an adaptive mechanism for moving intracellular lactate into the bloodstream. This provides an interesting comparison with the ^{31}P NMR studies of tumors, in which a similar type of mechanism could perhaps account for the unexpectedly normal value for the intracellular pH (58; and see above).

High resolution ^{1}H (34, 49, 102) and ^{13}C (4, 34, 49) NMR spectra have also been observed from the human forearm. The ^{1}H spectra show two main peaks, from water and fat, while the signals in the ^{13}C spectra are mainly from triglycerides. These nuclei can thus provide information about the composition and relative amounts of the tissue that is being examined. This is of value for studies of diseases such as Duchenne dystrophy, where it is important to know the volume of muscle tissue. In fact the ^{1}H spectra of patients with dystrophy show, as expected, an elevated ratio of fat : water

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signal (34, 79). In addition, the ^{31}P NMR spectra of these patients reveal a number of abnormalities, including reduced ratios of phosphocreatine to ATP and to inorganic phosphate (34, 79).

Studies have also been performed on isolated human kidneys. A clinical trial is in progress in which kidneys are routinely examined by NMR prior to transplantation. One aim of these studies is to correlate the metabolic state prior to transplantation with subsequent graft function, in the hope that the NMR spectra will provide a method of predicting early function. On the basis of the preliminary results, the outlook seems promising (19).

CONCLUSIONS

The development of NMR as a noninvasive method of studying metabolism is leading to a better understanding of the intracellular environment, metabolite levels, and enzyme activities within intact tissues. In many respects, NMR is complementary to the more traditional methods that are available for metabolic studies, and the various techniques are most informative when used in conjunction with each other. New information is emerging about metabolic control, the relationships between metabolic state and physiological function, and the relationships between enzyme activities in vivo and in vitro.

It seems likely that NMR will significantly enhance our understanding of human metabolism, both in healthy and diseased tissue. The technique could aid clinical diagnosis and could also be of value as an objective method of assessing the response of patients to therapy.

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