Contrast Agents and Spectroscopic Probes in NMR

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The demand for higher diagnostic specificity has led to the increased use of "foreign" agents to increase tissue contrast and/or spectroscopic sensitivity in NMR studies. The primary agents used to enhance tissue contrast in NMR imaging are paramagnetic. They cause a decrease in the proton T_1 of H_2O, leading to enhanced signal intensity. This effect depends on the large gyromagnetic ratio of the electron, the number of unpaired electrons, the concentration of paramagnetic ions, the number of coordinated water molecules, and the rate of exchange of water. Spectroscopic enhancement has relied primarily on attempts at isotopic enrichment (usually C-13), which causes a direct increase in signal.

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The preceding articles in this series (*1–3*) have shown how NMR can discern chemical and anatomical differences by relatively simple and direct methods. Initial clinical studies have demonstrated that ^1H NMR imaging has an excellent sensitivity in detecting a wide variety of lesions (*4–13*). The NMR relaxation parmeters, T_1 and T_2, provide almost all of the image contrast. Maximum tissue discrimination can be achieved by using different radiofrequency pulse sequences, as outlined in a previous paper of this series (*3*). In NMR spectroscopy, different metabolites can be identified by their peak positions. The presence of abnormal metabolites or chemical peaks shifted from their normal resonance position may indicate pathologic states. Note that the methods used in both techniques are inherently nonninvasive: the use of foreign agents or ionizing radiation is not needed.

However, major efforts are under way in the development and use of interventional methods to increase NMR image contrast and to visualize specific metabolite markers in spectroscopy. The need for contrast agents in NMR imaging arises because the relaxation times in different pathologic conditions (abscesses, malignant neoplasms, benign tumors) overlap and do not provide absolute specific diagnostic information (*14,15*). This low diagnostic specificity may be resolved as investigators develop more experience with NMR techniques and image interpretation. Even so, the ability to alter selectively the image intensity with paramagnetic agents may provide the needed diagnostic specificity in certain situations.

High-resolution NMR spectroscopy studies can also be expanded by the addition of "contrast" agents, i.e., by isotopic enrichment. These spectroscopic probes are often used to enhance the sensitivity of nuclear species (C-13 in particular) which otherwise could not be detected in vivo because of poor sensitivity or low natural abundance. As opposed to paramagnetic species, isotopic enrichment leads to a direct increase in signal observed from the sample. Isotopic enrichment and other spectroscopic probes that lead to a direct increase in signal will be discussed separately from image-enhancing agents, which consist primarily of paramagnetic ions.

IMAGE-ENHANCING AGENTS.

The mechanism by which paramagnetic agents provide NMR image contrast is fundamentally different from that induced by radiographic contrast media or radionuclide tracers. The last two are observed directly in radiographic or scintigraphic images, either by their ability to absorb x-rays or by emission of radiation. NMR contrast agents, however, operate in an indirect fashion, by altering the magnetic properties of the nuclei being observed in the image. Thus, the agents themselves are not detected but their effects are exerted by changing the NMR characteristics of the observed proton signal.

The effectiveness of an NMR imaging contrast agent

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depends on its ability to change the NMR properties [ρ (nuclear spin density), T_1, T_2] of the nucleus being studied. As mentioned previously, almost all clinical imaging has involved proton NMR, and the principal proton species that generates signal is the H_2O molecule. In general, the use of contrast-enhancing studies has been approached by reduction of the T_1 of H_2O by introduction of a paramagnetic species, and we will concentrate on this aspect of imaging contrast agents.

Diamagnetism and paramagnetism. Paramagnetic species affect relaxation rates ($1/T_1$ and $1/T_2$) because they possess electrons whose spins are "unpaired." Electron spin is analogous to the quantum-mechanical property of spin possessed by protons and neutrons. For most molecules, electrons are distributed in pairs into various "orbitals" (an orbital defines a probability of locating an electron at a position in space). The two electrons in a given orbital must have opposite spins (one up and one down), as described by the Pauli exclusion principle. Since these two spins cancel, there is no net electron spin associated with the molecule and it is said to be diamagnetic.

Some substances, however, have several orbitals at identical (or very similar) energy levels. In this case, only one electron may be placed into each orbital, all with parallel spins. A net electron spin results, and the substance is said to be paramagnetic. The unpaired electrons of paramagnetic agents generate local magnetic fields that shorten both T_1 and T_2 of neighboring protons, leading to a decrease in both relaxation parameters.

Referring back to our initial discussion on the effect of a magnetic field on a nuclear spin, one can understand that an unpaired electron spin will have a similar behavior. The magnetic moment (μ) of an unpaired electron is approximately 700 times that of a proton. Therefore the spin interaction between a proton and an electron (which is proportional to the product of the square of the individual values of μ) will be much stronger than that of a proton-proton interaction. It follows that paramagnetic agents are much more effective in enhancing relaxation times than nucleus-to-nucleus interactions. Close contact between the water nucleus and the paramagnetic species is required for this effect.

Typical paramagnetic species. The most common paramagnetic species in nature are metal ions, which usually possess incompletely filled d or f orbitals. Ions of these metals contain between one and seven unpaired electrons, as listed in Table 1. The strength of the magnetic moment of these species (which is one factor that determines the degree of relaxation enhancement) is roughly proportional to the number of unpaired electrons. Water molecules can bind directly to these ions, leading to a drastic decrease in the water relaxation times (increased relaxation rates).

Other paramagnetic species that have been used as NMR contrast agents include stable free radicals and oxygen. In the former group, the nitroxide free radicals may be useful because of their chemical versatility; these paramagnetic organic molecules, with one unpaired electron, can be covalently attached to a wide variety of substrates as "spin labels" (*16*). However, the water relaxation ability of these agents is quite low relative to some metal ions, so their use is limited to cases where relatively high concentrations ($1 - 10$ mM) of the free radical are achievable (*17*). Molecular oxygen is also paramagnetic, possessing two unpaired electrons, but its potential efficacy remains unknown.

TABLE 1.
MAGNETIC MOMENTS FOR VARIOUS PARAMAGNETIC METAL IONS*

Ion		Number of unpaired electrons	Magnetic moment (Bohr magnetons)
Ti^{3+}, V^{4+}, Cu^{2+}		1	1.7–2.2
V^{3+}		2	2.6–2.8
Ni^{2+}		2	2.8–4.0
Cr^{3+}, V^{2+}		3	~3.8
Co^{2+}	(high spin)†	3	4.1–5.2
Mn^{3+}, Cr^{2+}	(high spin)	4	~4.9
Fe^{2+}	(high spin)	4	5.1–5.5
Fe^{3+}, Mn^{2+}	(high spin)	5	~5.9
Gd^{3+}		7	8.0

* (From Drago RS: *Physical Methods in Chemistry*. Philadelphia, W.B. Saunders Co., 1977, Chap. 11).

† Number of unpaired electrons depends on chemical environment around metal ion. Thus, Table shows maximum number of unpaired electrons, which is referred to as high spin state. Under some conditions electrons can pair, giving low spin state, e.g., ferrous ions, (Fe^2) in the low spin state will have no unpaired electrons.

Factors influencing the water-relaxation ability of paramagnetic agents. It is easiest to discuss changes in T_1 due to paramagnetic ions by considering the factor $1/T_1$, which measures the rate of relaxation. Therefore, much of the following discussion will be related to the rate of relaxation, which is the inverse of relaxation time.

The effect of a paramagnetic agent on the relaxation rates of water protons can be described in an additive fashion:

$$\left(\frac{1}{T_1}\right)_{obs} = \left(\frac{1}{T_1}\right)_b + \left(\frac{1}{T_1}\right)_p,$$

where $(1/T_1)_{obs}$ is the observed longitudinal relaxation rate after adding the agent, $(1/T_1)_b$ is the baseline (or "before") relaxation rate of water or tissue, and $(1/T_1)_p$ is the additional contribution to the experimental rate caused by introduction of the paramagnetic species. A

similar relationship exists for the transverse relaxation rate, $1/T_2$. The magnitude of $(1/T_1)_p$ or $(1/T_2)_p$ (i.e., the change in relaxation rate caused by the paramagnetic ion) depends on several factors associated with the interaction between the unpaired electron and the water protons. These are discussed briefly here.

1. Concentration of the paramagnetic species. $(1/T_1)_p$ and $(1/T_2)_p$ are directly proportional to the concentration of the agent, with high concentrations providing drastic increases in the relaxation rates (or large decreases in the relaxation times). The concentration of most paramagnetic agents required to reduce tissue relaxation rates significantly is high (0.1 − 1.0 m*M*), especially when compared with the nanomolar to micromolar concentrations of radionuclides needed in gamma imaging. Because of the high concentrations required, we will need to develop nontoxic agents for clinical applications.

2. Magnetic moment of the paramagnetic species. The strength of the dipolar magnetic field caused by the unpaired electron is expressed in units of Bohr magnetons (μ_B), as shown in Table 1. Metal ions with multiple unpaired electrons tend to have higher magnetic moments (as high as 6–10 μ_B) than simple free radicals ($\sim$1.7 μ_B), so the relaxation enhancement from the former group tends to be larger.

3. Modulation of the magnetic interaction between electron and nucleus. This contribution to $(1/T_1)_p$ and $(1/T_2)_p$ is the most complex and hardest to visualize of all the important factors in paramagnetic relaxation effects. Only a cursory description will be offered here, since more complete treatments are available (*18,19*).

Nuclear relaxation is caused primarily by fluctuating magnetic fields stemming from the small magnetic dipoles of nuclei such as protons in water or the larger dipoles of the unpaired electrons in paramagnetic species. Fluctuations in these fields occur through some type of molecular motion (rotation, translation, or chemical exchange) or by the fluctuating electron-spin moment.

The rates of these fluctuations are characterized by a correlation time constant, τ_c, which is an important factor in determining the relaxation efficiency of a paramagnetic species. Generally, the larger the value of τ_c (slower electron—nuclear fluctuations), the higher the relaxation efficiency of the agent. Since τ_c can vary over many orders of magnitude (10^{-13} to 10^{-6} sec), the selection of species with large values of τ_c will undoubtedly be crucial in the design of powerful NMR contrast agents. τ_c can be varied by binding the paramagnetic species to a larger molecule like a protein, T_c. In addition, the characterization of correlation times in vivo will become increasingly important in order to understand and predict the magnetic alterations caused by these novel agents.

4. Number of coordinated water molecules. The increase in relaxation rate (decreased T_1) caused by a metal ion or complex is proportional to the number of water molecules coordinated directly to the metal ion, i.e., the solvation sphere. For aquo ions, usually six to ten water molecules are present. However, to decrease the toxicity and to help direct a metal ion to a specific tissue, the metal ion needs to be chelated with a suitable ligand. This unfortunately displaces at least a portion of the coordinated water molecules, generally resulting in lower relaxation efficiency.

5. Rate of water exchange. The paramagnetic effect of an unpaired electron is transmitted to the bulk water by exchange of water in the bulk phase with water molecules in the "solvation sphere"—i.e., the H_2O molecules attached to the paramagnetic ion. If the rate of exchange of H_2O between the solution and that bound to the paramagnetic species is very slow, (e.g., as for Cr^{3+}) one may see a diminished relaxation enhancement effect due to the less efficient communication of the paramagnetic effect to the bulk solvent.

6. Metal-to-nucleus distance. The relaxation enhancement of nearby nuclei falls off rapidly as the distance from the paramagnetic metal to the nucleus increases: the rates are proportional to $1/r^6$, where r is the metal-to-nucleus distance. The value of this parameter for water molecules coordinated to metal ions ranges between 0.26 and 0.31 nm, and only slightly modifies the observed rates.

It is clear from the above discussion that the design of effective paramagnetic agents is a difficult task and will probably require a concerted effort between chemists, biophysicists, and physicians.

Application of paramagnetic agents in NMR imaging. The development of contrast media for NMR is currently an area of great interest. The early progress is already documented in three recent review articles (*17,20,21*). We wish here to present only an overview of some of the various agents used and their potential applications.

Manganese (Mn^{2+}) has been used by several groups as an indicator of perfusion (*20,22–25*). Figure 1 displays some early results from our group using induced myocardial infarction in dogs. Images of the excised heart reveal significant contrast between normal and infarcted areas due to the presence of Mn^{2+} in the normal region of the myocardium.

Paramagnetic metal salts have also been used as oral contrast media. Ferric chloride (*26*) and the ferric ammonium citrate component in Geritol (*27*) have both been utilized in gastrointestinal imaging.

Metal complexes with various polydentate chelate ligands offer somewhat more promise, due to lower toxicity and target localization potential. Gd^{3+}-DTPA (DTPA = diethylenetriaminepentaacetic acid) and Cr^{3+}-EDTA (EDTA = ethylenediaminetetraacetic acid) have been used by various groups as flow agents or

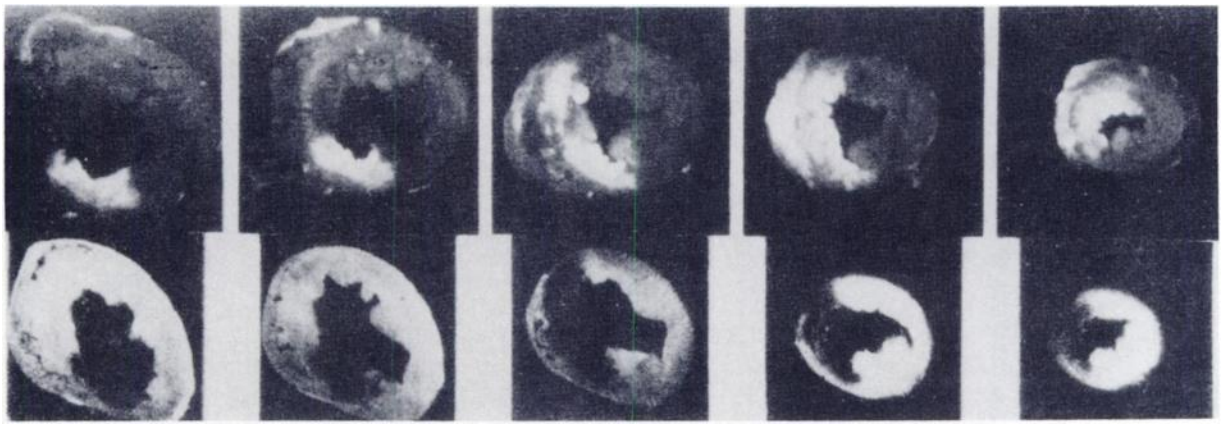

FIG. 1. Sections of dog's heart in which coronary artery had been ligated and $MnCl_2$ then infused. Bottom frame shows enhancement of NMR signal in steady-state free precession experiment (SSFP) in perfused areas, compared with ischemic ones. This confirms Mn^{2+} as short-term (<2 h) flow marker. Upper row of photographs shows hearts marked with tetrazolium vital stain, and one can see excellent correlation between NMR results and injury (from Ref. *22*).

general organ-contrast agents (liver and kidneys) (*21*).

Nitroxide free radicals have been utilized in renal (*28*) and brain (*29*) imaging. In volunteers breathing 100% oxygen, a decrease in the T_1 of blood in the left ventricle caused enhanced NMR signal intensity (*30*).

One of the more exciting uses of paramagnetic ions in NMR imaging is to direct them to specific tissues with monoclonal antibodies. A recent report (*31*) shows that the monoclonal antibody to cardiac myosin, which specifically localizes in infarcted myocardium, can be labeled with Mn-DTPA and used to enhance the infarcted region in NMR images. Similar agents directed toward specific human tumor lines or microbial species may be useful for the detection of neoplastic disease or abscesses.

Agents that alter properties other than relaxation time. There also exist ways to vary water content or replace water with another compound of different proton density or relaxation time. It is, of course, easily recognized from our third article (*3*) that a change in water content also will change relaxation times.

This area has few examples. Beale et al. have shown how diuretics and hormones can change water content in some tissue (*32*). It is also possible to introduce lipids into cavities like the intestinal tract. Two groups have shown that this can yield resolution enhancement due to both the lower relaxation time of lipids and their higher volume proton density (*32,33*). We also note that lipids have appreciable $—CH_2—$ concentration; such compounds may eventually be even better distinguished by the new technique of chemical-shift imaging (*34*).

SPECTROSCOPIC AGENTS.

Carbon-13. As mentioned in the second article of the series (*2*), C-13 nuclei comprise only 1% of the naturally occurring carbon nuclei. The addition of isotopically enriched C-13 compounds can bring the sensitivity of carbon NMR to the level achieved with phosphorus. It is therefore feasible to study cellular metabolism by feeding cells with C-13-labeled compounds (lactate, glucose, alanine, fatty acids, etc.) or perfusing organs with them, and following the C-13 label through the metabolic pathway (*35,36*).

The development of these techniques suffers from the same problems (primarily poor sensitivity) as did phosphorus NMR, and consequently has followed a similar course. Initially, studies were done on unicellular organisms (*37*), which were "fed" C-13-labeled compounds. Later Cohen et al. studied rat-liver cells that were fed C-13-labeled glycerol (*38*). Much of the early work has been done by Shulman's group (*39*). Subsequent studies have involved examination of isolated perfused organs (*40*). Surface coils have recently been used to study in vivo systems; in particular the storage of labeled glucose in rat liver, where it is stored as glycogen, has been investigated (*41*). One suspects that fatty acids will also be studied in vivo as they have in isolated organs.

These NMR studies show that past research was well carried out in that no startling new pathways have appeared, and most old ones demonstrated again, with the noninvasive elegant manner of NMR. This includes the reactions of the Krebs cycle and the glycolytic pathway.

Among the advantages of C-13-enriched compounds is that there is little background signal except in the fatty-acid region (see Fig. 5 in Ref. *2*) to interfere with the NMR experiment. Also, compounds can be synthesized where one or more positions are specifically labeled. It is therefore possible, for example, to study the fate of any particular carbon atom, C-1 through C-6, as glucose is oxidized.

A novel approach to analysis of the metabolic fate of labeled C-13 compounds (such as pyruvate, ethanol, or

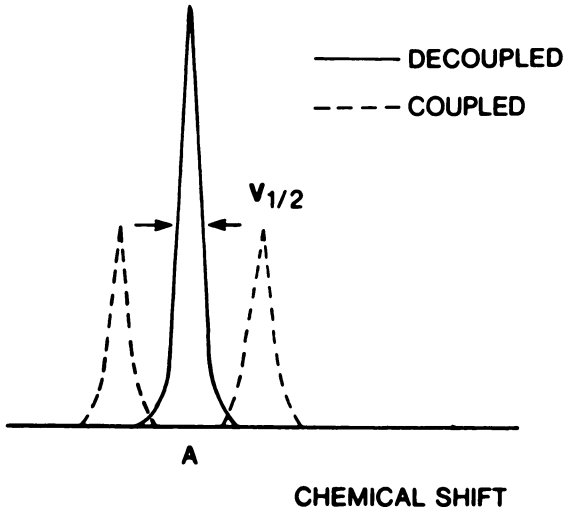

FIG. 2. Schematic of coupling of two nuclear spins. Coupled spectra show two peaks symmetrically displaced from resonance position when peak is decoupled (from Ref. *61*).

glycerol), and to prediction of the relative activities of different synthetic pathways, has been exploited by Cohen et al. They used the spin-spin splitting pattern (see Refs. *38,42* and Figs. 2 and 3) to study gluconeogenesis from C-13-labeled glycerol. They were able to distinguish glucose synthesized from unlabeled fructose from glucose synthesized from labeled glycerol by the different spin-spin splitting effect. They were therefore able to study the effect of exogeneous fructose on gluconeogenesis.

No studies have yet been reported on C-13 isotopic enrichment in humans. The primary problem is obtaining adequate amounts of C-13-labeled agent. Carbon-13-containing compounds are not easily isolated and can be very expensive: a typical price is $325.00 for one gram of 90% pure C-13 ethanol labeled at position C-1 (*43*). Considering the NMR sensitivity problem and the volume of C-13 compounds that might be required, careful studies in this age of cost-consciousness will be needed to establish whether the medical information obtained from NMR experiments can be balanced against expense.

There are additional implications to the fact that as C-13-containing compounds are metabolized, their label is chemically passed around. Since so many carbon-containing compounds exist, the C-13 label will appear in many compounds and the signal intensity diluted. How quickly this occurs will depend on the activity of the enzymes that use the compound, but quite complex spectra such as those in Fig. 4 can be generated. The information content of such spectra is high.

Fluorine-19. There has been some speculation on NMR applications of F-19-containing compounds, particularly fluorinated blood substitutes. However, most of the published studies have been on the fluorinated anesthetics (*44–46*).

The fluorinated anesthetics show significant alterations in their spectra in intact tissue, e.g., showing two peaks in a rat adenocarcinoma, compared with only one in normal rat kidney (*46*). Figure 5 demonstrates the point that anesthetics are lipid-soluble and therefore may provide a novel probe to study the state of membranes or other lipophilic environments.

Anesthetics do have several drawbacks. Their toxic range is in the low-millimolar level (*47*). The differences in peak position, as seen in Fig. 5, are generally less than 1 ppm. These small differences are hard to resolve in intact tissue and therefore will require relatively high S/N (i.e., long period of signal averaging) to inspire confidence in their reproducibility. However, their preliminary use suggests that it might be feasible to design and study similar lipophilic compounds that would concentrate safely in membranes at higher levels and with better spectral resolution in membranes.

Data have appeared containing F-19 NMR spectra of blood substitutes or other compounds that could serve as blood-flow and/or -pool markers (*48,49*). It is difficult to predict at this time how useful these compounds will be, due to paucity of detailed studies, but they can be

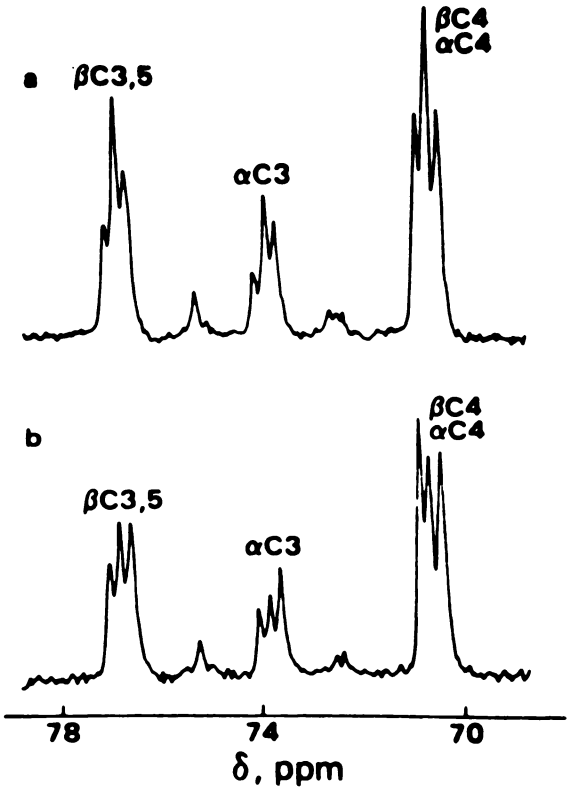

FIG. 3. C-13 NMR spectra of isolated liver cells. C-13-labeled glycerol (C_1 and C_3) was fed to cells. Labeled C-13 enters glucose at C_3 and C_4 positions, leading to doublet due to spin-spin coupling. In (a), fructose is added in addition to labeled glycerol, and is metabolized and incorporated into glucose as unlabeled entity. There is therefore no spin-spin splitting, leading to stronger central peak relative to (b), where only labeled glycerol is added (from Ref. *38*).

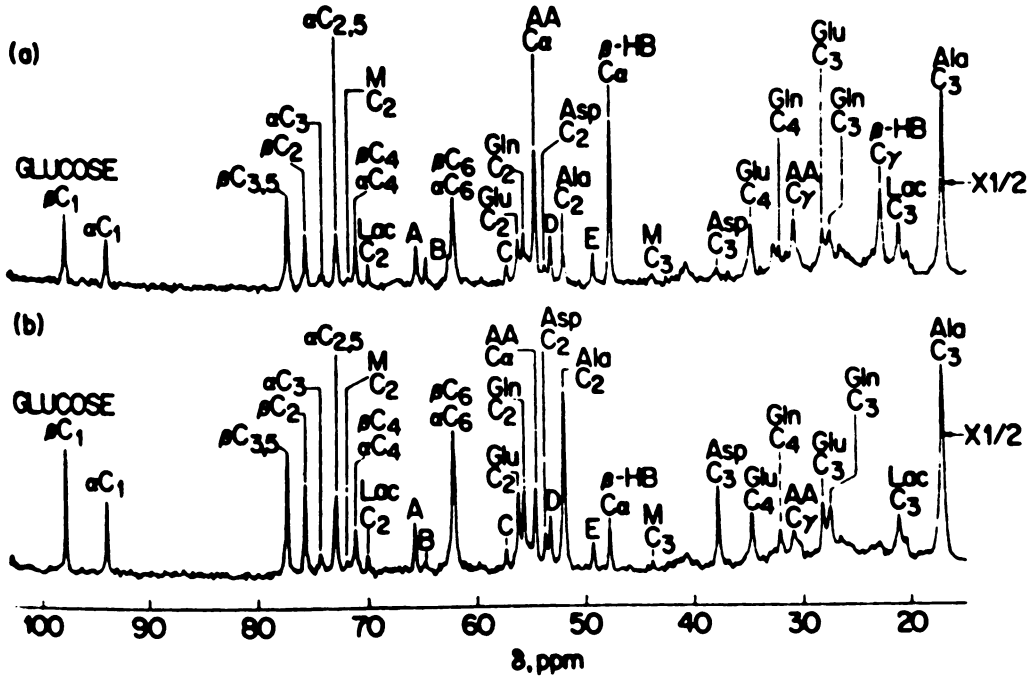

FIG. 4. Example of how molecular scrambling can generate many species as C-13 label is distributed among several compounds. Many resonances in spectrum all arose from few simple infused compounds (from Ref. *62*).

administered at much higher concentrations than anesthetics. Consequently, it is an area that should be carefully watched, since blood concentrations of fluorine in blood substitutes may approach levels where conventional imaging could be applied.

Other compounds that might be utilized are the same ones used in PET scanning, like fluorinated sugars, amino acids, and fatty acids. In NMR studies, the naturally abundant (almost 100%) isotope of fluorine, F-19, would be used instead of F-18. The problem, however, is that concentrations at least close to millimolar will be necessary for spectroscopic studies. At this concentration, toxicity is of real concern. Some plants from South Africa actually store fluorinated compounds as a protection against predators, since such compounds (generally fluorinated fatty acids) can be deadly (*50*). In isolated systems, fluorinated compounds have been observed (*51–53*).

Phosphorus-31. Few spectroscopic agents containing phosphorus have been used in augmenting the interpretation of P-31 NMR spectra. Those that have been are in the phosphonate family, containing a P-C bond in contrast to the usual P-O bond of the phosphates. Phosphonates are familiar in nuclear medicine in such carrier compounds as methylene diphosphonic acid. However, methylphosphonate has been transported intracellularly by *E. coli* (*54*) and erythrocytes (*55*). Also, phosphonocholine, an analog of choline, has been transported by cells and eventually incorporated into their membranes (*56*). Whether such compounds can be used in mammalian systems is an additional area for new pharmacological research.

There are also several examples in the literature of other compounds that can be transported into the cell and then phosphorylated. In the second article of our series 2-deoxyglucose has already been discussed (*2*). Another class of such compounds is the analogs of creatine, like cyclocreatine. It is potentially feasible to measure creatine uptake and phosphorylation with them (*57*), since differences between phosphocreatine and phosphoarginine can easily be measured (*58*).

Other nuclei. There are several other nuclei that could be incorporated into biologic compounds and might then be observed in vivo. In particular these include Na-23, H-2, K-39, N-14, N-15, Mg-25, and Ca-43. Examination of Table 1 in the first article (*1*) shows why these are much less likely to find any routine spectroscopic use. These nuclei are either not sensitive enough, are of too low natural or biological abundance, are too expensive,

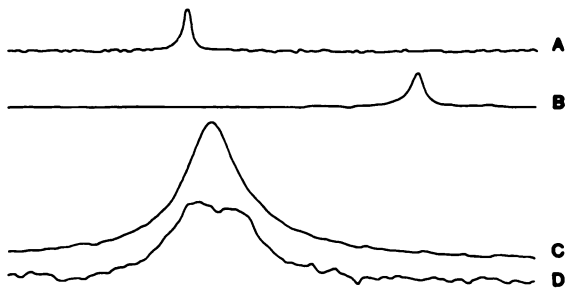

FIG. 5. Fluorine spectra from halothane (CF_3-CHBrCl) dissolved in water (A), in hexane (B), in equilibrated rat tissue such as normal kidney (C), or in adenocarcinoma of kidney (D). Tissues have been removed and examined in vitro. Double peak is observed in tumor compared with normal kidney. (From Ref. *46*.)

or possess NMR properties that make them unattractive for ready application. However, the high extracellular Na-23 concentration has been useful for imaging (*59,60*).

Spectroscopic probes that would be of use in the study of cell biochemistry are still very much in the developmental stage. This is not unexpected, since in vivo spectroscopic studies are themselves only a few years old. These techniques will undoubtedly be exploited further over the next few years as more sophisticated studies of cellular metabolism are designed.

SUMMARY

Agents that may be added externally to augment either spectroscopic or imaging studies have now begun to appear. In the spectroscopic area, C-13-containing compounds have been the main agents of choice, although fluorine and compounds that can be phosphorylated are also of interest. For imaging studies, agents that affect relaxation time, such as paramagnetic ions or stable free radicals, have been most actively studied. The effect of paramagnetic ions on tissue is a complex theoretical one, and it is probable that clinical applications will outstrip theoretical explanations.

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