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PREPARATION OF A MODIFIED Gd-DTPA COMPLEX: A POTENTIAL
CONTRAST AGENT FOR MAGNETIC RESONANCE IMAGING

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Thomas Hudson Moss III

June 1986

DEDICATION

This author dedicates this thesis to his parents, Tom and Rita Moss, as they have so unselfishly dedicated their lives to their children.

ACKNOWLEDGMENT

The author wishes to express appreciation to Dr. George W. Kabalka for his guidance. Also the author wishes to thank Dr. Leaf Huang and Nicky Norley for their assistance and collaboration in the project. Financial support was furnished by the Department of Energy Grant No. DE-FG05-86ER60434.

ABSTRACT

Magnetic Resonance Imaging (MRI) of the liver and spleen has yet to equal the quality of MRI generated from the central nervous system. Gd-DTPA is very effective at reducing the spin-lattice relaxation times of the hydrogen in cellular water and is thus a good contrast enhancing agent. However, Gd-DTPA is not tissue specific and is indiscriminately distributed throughout the vascular system of most tissues. The subject of this thesis is the development of a Gd-DTPA derivative that accumulates specifically in the liver and spleen and reduces the spin-lattice (T_1) relaxation times of those organs.

DTPA was covalently linked to stearylamine and the resultant ligand was complexed to gadolinium ions to form a paramagnetic phospholipid analog. The new complex was incorporated into the lipid bilayer of liposomes as a means for delivery. Normal Balb/c mice received intravenous injections of the liposomal suspensions and the spin-lattice relaxation times of the liver and spleen were measured in vitro. The relaxation rates of the livers and spleens were increased by 100 and 67%, respectively, within 30 minutes after administration of 14 μ moles of lipid/mouse (0.22 mmole Gd/kg). Biodistribution studies revealed that, within 30 minutes, 44% of the injected dose accumulated in the liver and 12% in the spleen. After 24 hours, 70% of the administered dose appeared in the liver compared to 21% in the spleen.

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CHAPTER I

INTRODUCTION

A. NMR THEORY

Nuclear Magnetic Resonance (NMR) spectroscopy has become a valuable analytical tool to chemists, biochemists, biologists, and, most recently, to physicians since the first observation of the NMR phenomenon by Purcell¹ and Bloch². Chemists use NMR to deduce molecular structure, to probe chemical and physical properties of molecules, as well as to monitor reactions. Biologists and biochemists utilize NMR to gather information on cell physiology, cell membrane transport mechanisms, photosynthesis, and to study metal ion binding to biological macromolecules. More recently, in the field of medicine, NMR has proven to be an important diagnostic imaging modality much like computerized tomography (CT) and positron emission tomography (PET) with the advantage that ionizing radiation is not required. It is now possible to utilize NMR signals of the hydrogen in human tissues (water, fats, muscle, etc.) to generate images of the type shown in Figure 1.

There have been many exhaustive treatments of the theory of the NMR phenomenon.³⁻⁵ Although a rigorous treatment is beyond the scope of this thesis, an understanding of the basic principles of NMR is important to the appreciation of its applications in medicine, as well as chemistry and biochemistry. Only those nuclei that have nuclear spin can undergo the NMR phenomenon. All nuclei possessing spin

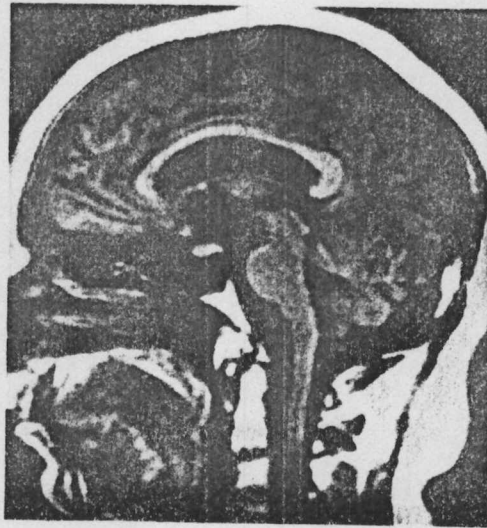


Figure 1. Sagittal MR image of the head. Generated from the water hydrogen signal. [From L. T. Bilaniuk, F. W. Wehrli, R. A. Zimmerman et al., Radiology, 153, 412 (1984).

contain either an odd number of protons, an odd number of neutrons, or both. Consequently, almost all elements possess magnetically active isotopes. The spin of a nucleus is represented by the nuclear spin quantum number, I . If the mass number is odd, the nuclei have I values that are integral multiples of $1/2$, i.e., $I = n/2$. Nuclei with even mass numbers and even atomic number have no spin, thus $I = 0$. Nuclei with even mass numbers and odd atomic numbers have integral values of I or $I = 1, 2, 3, \dots$ etc. There are many nuclei that may be considered for NMR spectroscopy but this discussion will focus on the hydrogen nucleus, $I = 1/2$, because of its relevance to this thesis.

Magnetic Properties of the Proton

The proton, being a positively charged, spinning body, has magnetic properties. In addition, because it is spherical, the proton has no electric quadrupole moment. In the presence of an applied external magnetic field, H_0 , the nucleus has a magnetic moment vector μ_N parallel to the nuclear angular momentum vector J_N and behaves like a tiny bar magnet with North and South poles.

The magnetic moment vector is given by the equation

$$\mu_N = g_N (e/2m_p c) J \quad (1)$$

where g_N = nuclear g-factor

e = electron charge

m_p = proton rest mass

c = the speed of light.

J is the angular momentum given by the equation

$$J = [I(I + 1)]^{1/2} \hbar \quad (2)$$

where $\hbar = h/2\pi$; h = Planck's constant.

Equation 1 can be rewritten as

$$\mu_N = g_N (eh/2m_p c) \hbar^{-1} [I(I + 1)] \hbar . \quad (3)$$

The expression $eh/2m_p c$ is called the Bohr magneton B_N . Substitution gives the expression of the magnetic moment as

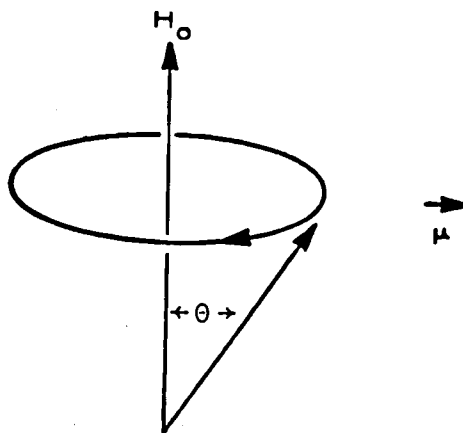
$$\mu_N = g_N B_N \hbar^{-1} J . \quad (4)$$

The g_N and I parameters distinguish one nucleus from another. For the proton, $g_N = 5.8555$ and $I = 1/2$ to give a value of 1.4109×10^{-23} ergs/gauss for the magnetic moment.

The applied magnetic field imposes a torque, T , on the magnetic moment that is equal to the rate of change of momentum with respect to time and is dependent on the magnetic field strength as described by the equation

$$T = \mu H_0 = dJ/dt. \quad (5)$$

As a result, the magnetic moment vector precesses about the axis of rotation in the direction of the applied external magnetic field at an angle θ .



The rate at which the magnetic moment vector precesses is called the Larmor frequency ω_0 given by the equation

$$\omega_0 = \gamma H_0 \quad (6)$$

Where γ is the gyromagnetic ratio given by

$$\gamma = g_N B_N \hbar^{-1} \quad (7)$$

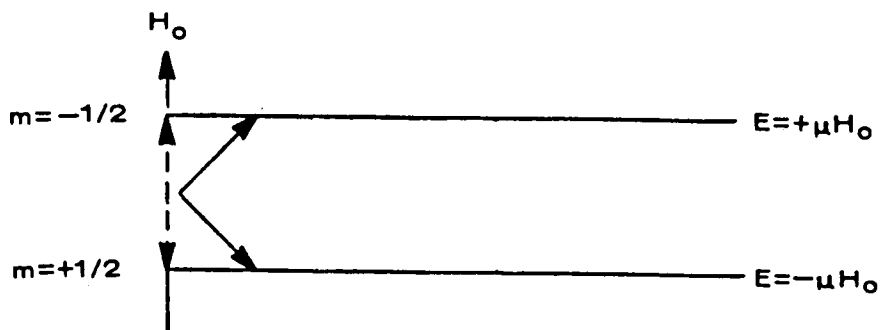
The gyromagnetic ratio is the ratio of the magnetic moment to the angular momentum.

Resonance Condition

A nucleus in a magnetic field can have $2I + 1$ values of magnetic quantum number, m_I , corresponding to $2I + 1$ possible projections of the angular momentum vector along the Z-axis. These values range from $-I$ to $+I$ in integral multiples according to quantum mechanical selection rules. For the proton there are two such energy states for which

$m_I = +1/2$, the lower energy state, and $m_I = -1/2$, the higher energy state. The energy E of the nucleus in the m_I state is given by the equation

$$E = -\mu_N H_0 . \quad (8)$$



Since the angular momentum and magnetic moment vectors can assume only certain orientations defined by quantum mechanical selection rules, the Z-component of these vectors can have only certain values defined by m_I . The energy difference between the lower energy state, which corresponds to a parallel alignment with H_0 , and the higher energy state, which corresponds to the antiparallel alignment, is given by

$$E = (\mu_N/I)H_0 . \quad (9)$$

For the proton, $I = 1/2$, the energy difference is $2\mu_N H_0$.

This energy difference is small compared to that of electronic energy states. As a result, the excited nuclear state, $m_z = -1/2$, may be highly populated at room temperature. The ratio of the population

in the excited state (N^*) to that in the ground state (N) is governed by the Boltzman distribution.

$$N^*/N = e^{-\mu_N H_0 / kT} \quad (10)$$

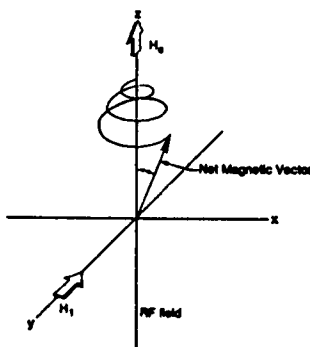
$$= e^{-E/kT} \quad (11)$$

where k = Boltzman constant, and

T = Temperature.

With an applied field of 14.09 KG, at room temperature, this ratio is 0.9999904. This is equivalent to an excess of only 9.6 nuclei out of every one million in the ground state. Hence, saturation of the excited state is facile. Therefore, there must be some means for the excited nuclei to return (relax) to the ground state. These relaxation processes are important and will be discussed later.

If an oscillating magnetic field H_1 is introduced perpendicular to the applied field such that the oscillating field is at the Larmor frequency, resonance will occur, and the spins will "flip." Consider a magnetic moment vector precessing about H_0 .



From A. Zermeno in "Nuclear Magnetic Resonance," R. C. Hickey, Ed., Yearbook Medical Publishers, Inc., Houston, Texas, 1982, p. 14.

If H_1 is applied along the y-axis, the spin system will experience a new magnetic field $H_{\text{eff}} = H_0 + H_1$, and the spin will attempt to precess about H_1 . As a result, the angle of precession about H_0 will increase as the vector spirals down toward the x-axis. The extent to which θ increases is determined by the time, t , that H_1 is applied. H_1 is usually applied as a radio frequency (Rf) pulse, and the time that H_1 is applied is called the pulse width (PW). The rate of precession following the Rf pulse, ω_1 , is given by the change in θ per unit time.

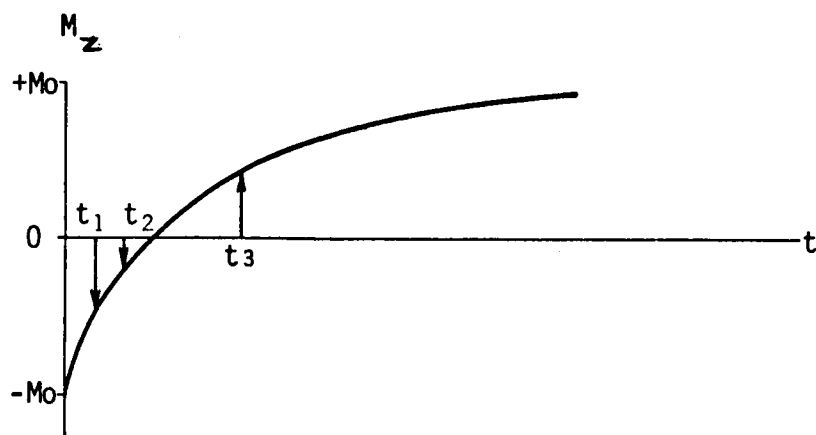
$$\omega_1 = \theta/t \quad (12)$$

We will be most concerned with those values of t which result in 90° and 180° spin flips.

Relaxation Mechanisms

As mentioned earlier, the energy difference between the two energy states for the proton is small, resulting in facile saturation of the excited state. In order to avoid saturation, there must be some relaxation mechanism for the transfer of energy and return to the ground state. Two important relaxation mechanisms are longitudinal (T_1) and transverse (T_2) relaxation. We will be most concerned with the longitudinal relaxation.

The longitudinal relaxation is the return of the M_z magnetization vector to the thermal equilibrium value M_0 along the z-axis following perturbation.



Relaxation occurs as the result of the exchange of energy with the lattice, which constitutes the thermal reservoir. Thus the longitudinal relaxation is termed the spin-lattice relaxation. It is also referred to as thermal relaxation. The decay of M_z is logarithmic and is described by a portion of the Bloch⁶ equations:

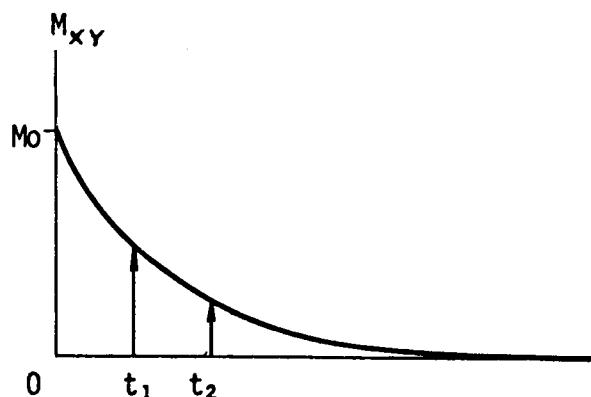
$$\frac{dM_z}{dt} = \frac{M_0 - M_z}{T_1} \quad (13)$$

The exchange of energy with the environment is the result of the interaction of the magnetic moment of the nucleus of interest with fluctuating magnetic fields in the environment.

Fluctuating magnetic fields are generated in part by molecular motions. Bloembergen, Purcell, and Pound⁷ made use of the theory of Brownian motion of molecules to describe the spin-lattice relaxation mechanism. The major pathways in which molecular motions give rise to fluctuating magnetic fields are (1) fields due to magnetic moments of

other nuclei (intra- and intermolecular), (2) fields due to variable electronic screening of the static field, and (3) fields associated with the spins of unpaired electrons. The last pathway is the most important contribution if paramagnetic species are present as will be discussed later.

The transverse relaxation process involves the decay of the magnetization vector M_{xy} in the xy -plane to the equilibrium value $M_{xy} = 0$ following perturbation.



The M_{xy} vector is made up of components of M_x and M_y . The decay of M_{xy} is the result of the dephasing of the M_x and M_y components. The dephasing results from the interactions of the spins of the nuclei and, hence, the transverse relaxation is termed spin-spin relaxation. Inhomogeneity of the applied field also causes dephasing of the spins. The decay of M_{xy} is given by the following portion of the Bloch⁶ equations:

$$dM_x/dt = -M_x/T_2 \quad (14)$$

$$dM_y/dt = -M_y/T_2 \quad (15)$$

B. PRINCIPLES OF IMAGING

Most NMR images today are generated from the signal of the hydrogen in the water of tissues.⁸⁻¹⁰ The human body is approximately 80% water, and the signal from the water hydrogen is a simple, single peak. In addition, hydrogen has a very high relative receptivity, 1.00¹¹ (relative to 1.76×10^{-4} for ^{13}C). Other nuclei that might be considered for image production are ^{31}P , ^{13}C , ^{15}N , which are constituents of many diagnostically important biomolecules such as ATP, ADP, phosphocreatine, glucose, etc., and ^{19}F . However, these nuclei are not as promising as hydrogen for NMR imaging using current technologies as a result of either their low biological abundance, relative receptivity, isotopic abundance, or a combination of each as illustrated in Table 1.^{11,12} In addition, the signals produced by the molecules mentioned above are often complex and difficult to interpret. These factors lead to long imaging times, which give rise to poor resolution due to patient movement.

In normal NMR spectroscopy, a homogeneous magnetic field over the entire volume of sample is essential. Recall that the Larmor equation reveals the relationship of the resonance frequency to the magnetic field strength.

$$\omega_0 = \gamma H_0 \quad (6)$$

As a result, nuclei with the same Larmor frequency will resonate at the same frequency in a given magnetic field. Hence, only one signal will

Table 1. Nuclei of Interest for Image Production

Isotope	% Biological Abundance ^a	% Natural Isotopic Abundance	Relative Receptivity ^b
¹ H	10	99.985	1.00
³¹ P	1.0	100	0.0663
¹³ C	0.19	1.108	1.76×10^{-4}
¹⁵ N	0.01	0.37	3.85×10^{-6}
¹⁹ F	Trace	100	0.8328

^a% by wt; % Biological Abundance = % natural isotopic abundance x % biological abundance of the element.

^bRelative to ¹H.

be obtained from the hydrogens in water, or any chemically equivalent nuclei. Also, the resonance frequency of water is identical in every cell regardless of the organ from which the signal is obtained. Therefore, there must be some means to differentiate or spatially resolve those signals that arise from different anatomical positions in order to generate images that yield useful diagnostic information.

Spatial encoding is obtained by introducing sequences of gradient magnetic field pulses which produce volume elements called pixels;¹³ each pixel has a unique field strength according to its position. As a result, more than one signal may be obtained from a sample of water according to its position within the magnetic field. The number of signals generated is dependent upon the number of pixels generated. The more pixels generated, the more signals obtained, and hence, the better the image resolution. Currently, a resolution of 1 mm has been obtained.¹⁴

Other than instrumentation, the images generated from the signal of cellular water are dependent upon four factors:¹⁵

1. Pulse sequence
2. Proton density
3. Spin-lattice and spin-spin relaxation times
4. Flow rates.

The hydrogen density, T_1 , T_2 , and flow rates are all intrinsic to the nature of the tissue, whereas the pulse sequence is not. It has been well documented that the relaxation times of normal and diseased

tissues are different.^{16,17} However, in some cases the relaxation times of diseased tissues may overlap with normal tissues resulting in poor resolution.¹⁸

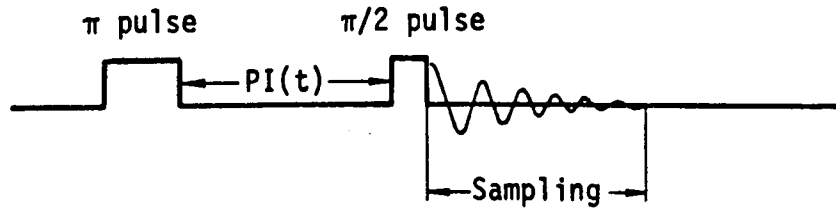
Tissues have different T_1 and T_2 values depending on their environment and physiological state. Depending on the pulse sequence used, hydrogens that have relatively short T_2 's may give weak signals and, thus, appear dark in an image as a result of the loss of transverse magnetization before the signal can be observed.¹⁹ Also, hydrogens with relatively long T_1 's can be easily saturated. As a result, such hydrogens also give weak signals if the pulse repetition rate is too high. In many instances the T_1 and T_2 parameters of different tissues overlap making contrast poor. Recently there has been an intense interest in the development of pulse sequences and pharmaceutical agents that will alleviate these problems.^{15,20}

C. CONTRAST ENHANCEMENT VIA PULSE SEQUENCING

The most common sequences used in magnetic resonance imaging (MRI) are the inversion recovery (IR) and spin-echo (SE) sequences. Depending on the tissue to be investigated, one must determine which sequence to use. The IR sequence gives T_1 weighted images. The SE sequence can give either T_1 or T_2 weighted images.

Inversion Recovery

The IR sequence consists of a 180° pulse followed by a 90° pulse and is represented as $180^\circ - \tau - 90^\circ$.¹⁹



The time between pulses is the pulse interval PI denoted by τ . The 180° pulse inverts the magnetization onto the negative z -axis. The magnetization M_z immediately begins to relax back to the equilibrium value $M_z = M_0$ by the exponential T_1 process. After a time, τ , a 90° sampling pulse is applied which flips the remaining magnetization into the xy -plane. At $PI = 0.693 \times T_1$, no signal will be observed. This point corresponds to the intersection of the signal intensity line on the time axis in the diagram on page 9. In order to obtain a strong signal, the PI must be kept short or long relative to the T_1 . However, if the sequence is to be repeated a number of times, there must be a sufficiently long ($5 \times T_1$) delay time PD between each pulse sequence to avoid saturation of the signal. The relationship between signal intensity S , PI , and PD is given by the equation¹⁹

$$S = M_0(1 - 2 \cdot \exp(-PI/T_1) + \exp(-PD/T_1)) \quad (16)$$

where M_0 is the equilibrium magnetization vector which takes into account the proton density. Thus, by varying the PI and PD parameters, greater contrast between tissues with different T_1 's can be obtained as illustrated in Figure 2.

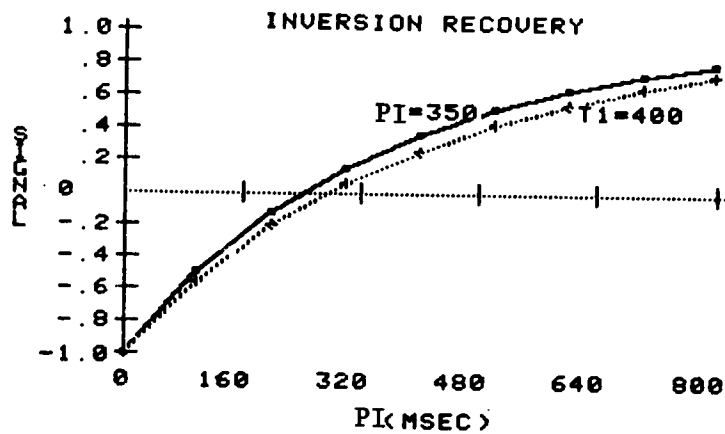
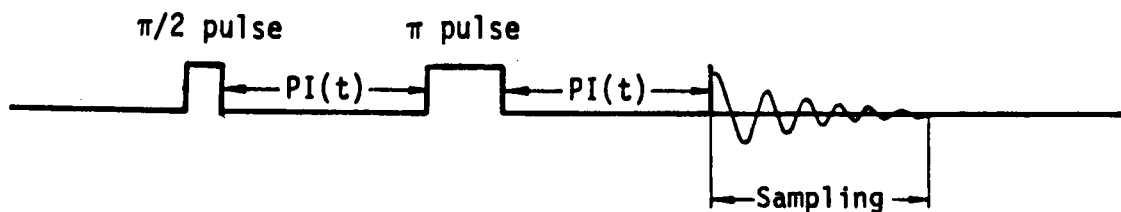


Figure 2. Contrast enhancement via IR pulse sequence. The signals from two tissues with $PI = 350$ and 400 msec, respectively, in an inversion recovery sequence assuming M_0 is the same for both. [Edited from J. C. Gore in "Nuclear Magnetic Resonance and Correlative Imaging Modalities," C. L. Partain, ed., The Society of Nuclear Medicine, Inc., New York, NY, 1984, p. 83]

The figure represents signals obtained from two different tissues having T_1 's of 350 and 400 ms. Notice that at $PI = 0.693 \times 350$ ms no signal is obtained from the tissue having the shortest T_1 , but the tissue with the longer T_1 gives a strong signal. Likewise, when $PI = 0.693 \times 400$ ms no signal is obtained from the tissue having the longer T_1 , and the other tissue gives a strong signal. Thus, by simply varying the PI parameter, an image of considerable contrast may be generated. Although at certain values of PI the signal will be negative, only the absolute signal intensity is usually computed in image production.

Spin Echo Sequence

The spin echo sequence consists of a 90° pulse followed by a 180° rephasing pulse and is represented as $90^\circ - \tau - 180^\circ$.¹⁹



The time between the pulses, τ , is PI, and the time between the 90° pulse and observation of the signal is the echo time $TE = 2 \times \tau$. The 90° pulse flips the magnetization vector into the xy-plane. The spins immediately begin to dephase as the result of spin-spin interactions (T_2) and field inhomogeneity effects (T_2^*). After a time, τ , a 180° rephasing pulse is applied that flips the spins into the negative

xy-plane as described in Figure 3. The dephasing continues in the same direction following the 180° pulse. As a result, at time $2 \times \tau = TE$, an echo is observed that is diminished in intensity as the result of the T_2 process.

If the sequence is repeated infrequently (i.e., PD is long relative to T_1), T_2 dependent images can be obtained. If the sequence is repeated relatively rapidly such that partial saturation is introduced, T_1 dependent images can be generated. The relationship between signal intensity, and the pulse and relaxation parameters is given by the equation¹⁹

$$S = M_0 \cdot \exp(-TE/T_2) [1 - \exp(-PD/T_1)] . \quad (17)$$

Thus by altering the TE and PD parameters correctly, image contrast can be produced where the T_1 and T_2 parameters are similar as illustrated in Figure 4. Here the PD is held constant at 200 ms, which produces partial saturation, and TE is varied. The tissues have T_1 's of 200 and 400 ms. It is assumed that M_0 is the same for both tissues. Large differences in signal intensities reflect good contrast.

D. CONTRAST ENHANCEMENT VIA CHEMICAL AGENTS

Recently there has been intense interest in the development of pharmaceutical agents that will alter the intrinsic parameters for imaging, namely the spin-lattice and spin-spin relaxation rates, and, to a lesser extent, proton density.²⁰ Organs of the viscera, an area of interest in most imaging modalities, are all very similar in proton

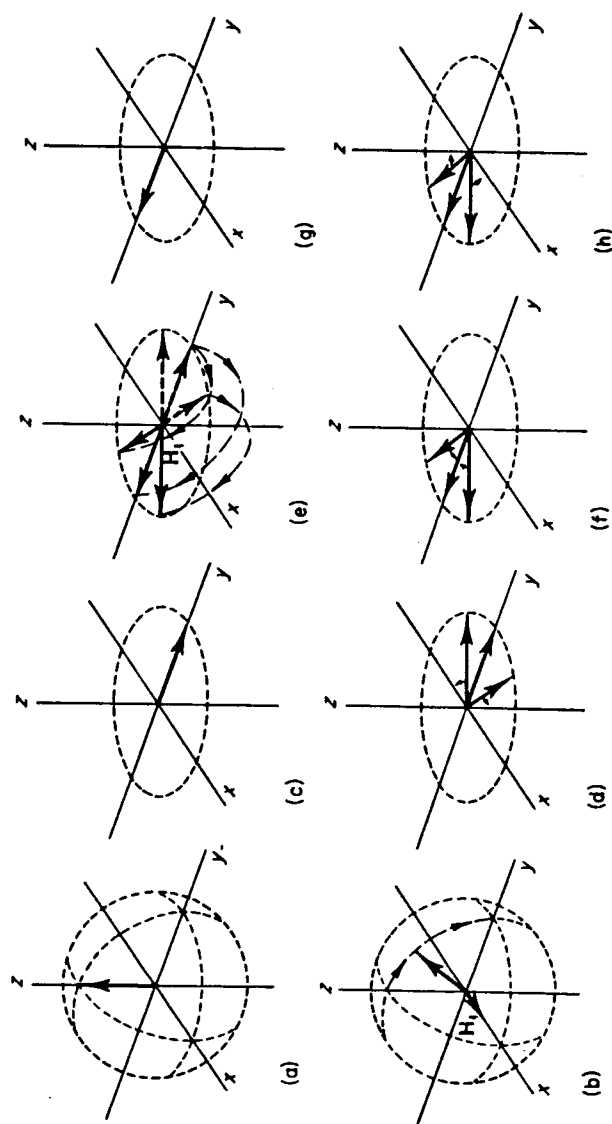


Figure 3. The formation of an echo. Initially the net magnetic moment vector is in its equilibrium position (a) parallel to the direction of the strong external field. The rf field H_1 is then applied. As viewed from the rotating frame of reference the net magnetic moment appears (b) to rotate quickly about H_1 . At the end of a 90° pulse the net magnetic moment is in the equatorial plane (c). During the relatively long period of time following the removal of H_1 , the incremental moment vectors begin to fan out slowly (d). This is caused by the variations in H_1 over the sample. At time $t = \tau$ the rf field H_1 is again applied. Again the moments (e) are rotated quickly about the direction of H_1 . The time H_1 is applied just long enough to satisfy the 180° pulse condition. This implies that at the end of the pulse all the incremental moment vectors begin to recluster slowly (f). Because of the inverted relative positions following the 180° pulse and because each incremental vector continued to precess with its former frequency, the incremental vectors will be perfectly clustered (g) at $t = 2\tau$. Thus maximum signal is induced in the pickup coil at $t = 2\tau$. This maximum signal, or echo, then begins to decay as the incremental vectors again fan out (h). [From H. Y. Carr and E. M. Purcell, Phys. Rev. 94, 630 (1954)].

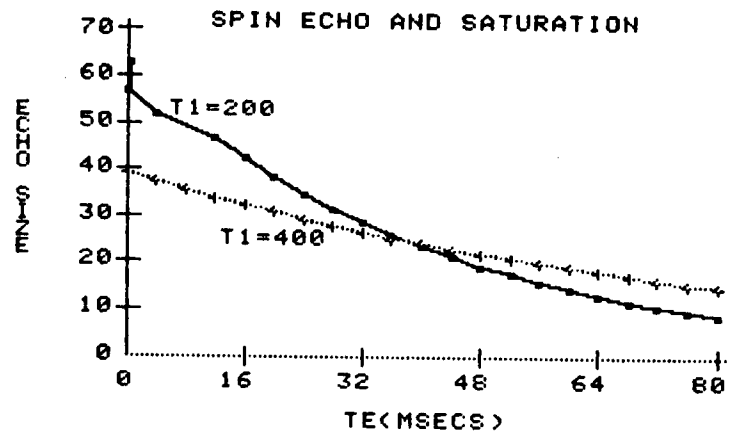


Figure 4. Contrast enhancement via SE pulse sequence. Variation of echo sizes with TE for two tissues with $T_1 = 200$ and 400 and $TR = 200$. $T_1/T_2 = 4$ and M_0 is the same for both tissues. [From J. C. Gore in "Nuclear Magnetic Resonance and Correlative Imaging Modalities," C. L. Partain, ed., The Society of Nuclear Medicine, Inc., New York, NY, 1984, p. 81]

concentration. There have been attempts to alter the proton density by administration of lipid solutions, olive oil, and ethanol. However, no significant change in signal intensities can be induced by such agents at safe dosages. Therefore, attention has focused on the utilization of paramagnetic species for image contrast enhancement.

Block, Hansen, and Packard²¹ first showed that paramagnetic ions drastically reduced the spin-lattice relaxation times of the protons in water. Paramagnetic ions contain unpaired electrons. An unpaired electron has a magnetic moment approximately three orders of magnitude greater than that of a proton. Hence the fluctuating magnetic fields associated with the unpaired electrons of ions tumbling in solution override the nuclear dipole interaction of protons for relaxation. The relationship between T_1 and the magnetic moment of a paramagnetic molecule is given by the equation⁷

$$\frac{1}{T_1} = \frac{12\pi^2 \gamma^2 \eta N_p \mu_{\text{eff}}^2}{5kT} \quad (18)$$

where η = viscosity of the solution

N_p = concentration of the paramagnetic molecules

μ_{eff}^2 = the effective mean square magnetic moment of the ion

k = Boltzmann constant

T = temperature ($^{\circ}\text{K}$)

This relationship is very important in the design of contrast agents. In particular, note the direct dependence of the relaxation rate ($1/T_1$) on the square of the magnetic moment of the ion and its concentration.

Hence the relaxation rate can be increased by increasing the concentration of the ion or by utilizing an ion with a larger magnetic moment.

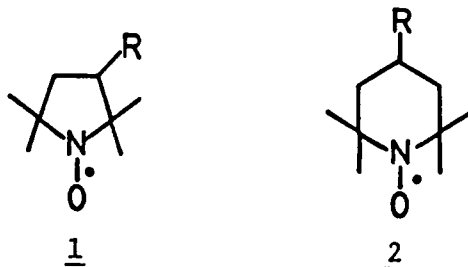
The equation above also takes into account the correlation time, τ_c , which is the time it takes for a molecule to experience a different magnetic field as a result of its tumbling. It is given by the equation

$$\tau_c = 4\pi \eta a^3/kT \quad (19)$$

where a is the radius of the molecule represented as a sphere. Bloembergen⁴ showed, with glycerin, that as the correlation time approaches the Larmor frequency, the frequency of fluctuating magnetic fields will most affect the relaxation rate. Hence, a possible approach to altering relaxation rates is to alter the correlation time. Reuben²² found that complexing gadolinium ions to serum albumin enhances the relaxation rate of water relative to the relaxation rate in the presence of free gadolinium ions as a result of the change in the correlation coefficient of the molecule.

Inhalation of molecular oxygen is a simple approach to altering the T_1 of blood. This method has been found to decrease the T_1 of the blood in the left ventricular cavity of the heart in volunteers.²³ It may be possible to use the contrast enhanced images produced by O_2 inhalation as a means to monitor metabolic processes. However, oxygen is rapidly metabolized in vivo resulting in the rapid loss of paramagnetic properties.²⁴

Another class of paramagnetic species that has been investigated for MRI is the nitroxide stable free radical (NSFR) or nitroxide spin labels (NSL). These pyrrolidnyl 1 and piperidinyl 2 derivatives have an unpaired electron localized in the nitrogen-oxygen bond. Substitution of methyl groups at the carbon alpha to the nitrogen renders the free radicals stable toward disproportionation.



They are readily covalently linked to biomolecules by appropriate alterations of the -R groups. Increased relaxativity has been demonstrated by increasing the atomic weight by binding to plasma.²⁵ NSL's are rapidly removed by the kidney at the rate of glomerular filtration. They have been evaluated as agents for the assessment of renal function,^{26,27} myocardial infarction,²⁸ and subcutaneous neoplasms.²⁷ However, nitroxide spin labels are rapidly reduced to noneffective diamagnetic N-hydroxylamines upon first pass through the lungs.²⁹ Thus, their use as MRI contrast agents has been limited.

A third and more important class of paramagnetic species are metal ions of the transition and lanthanide series. First row transition metal ions contain partially filled 3d orbitals. By virtue of Hund's rule, the five degenerate 3d orbitals are filled so as to give a maximal spin angular momentum. As a result, metal ions Mn^{2+} , Cr^{3+} , and

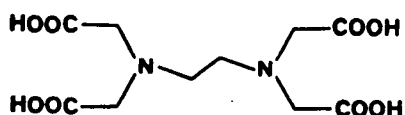
Fe^{3+} , which have 5, 3, and 5 unpaired electrons respectively, are promising species for MRI contrast agents due to their large magnetic moments. Also, metal ions of the lanthanide series with partially filled 4f orbitals are attractive possibilities. Of the lanthanides, Gd^{3+} with seven unpaired electrons has proven to be most effective.

Solutions containing free Fe^{3+} and Fe^{2+} have been successfully utilized for gastrointestinal imaging. Young et al. found that oral administration of 0.06% ferric chloride solution decreased the T_1 of the fundus of the stomach.²³ Wesbey and coworkers utilized dietary ion supplements containing ferric ammonium citrate and ferrous sulfate hexahydrate for the evaluation of their efficacy in gastrointestinal imaging.³⁰ They found that these agents produce good declination of the bowel loops. Solutions of MnCl_2 have shown potential in heart imaging.³¹ Manganese ions accumulate in the myocardium and hepatic cells. In an acute myocardial infarction model, no detection of the ischemic area was observed prior to administration of MnCl_2 . However, after the administration of MnCl_2 the ischemic tissue was clearly delineated from the healthy myocardium. MnCl_2 has also been found to decrease the T_1 of the liver.³² GdCl_3 solutions have been found to decrease the T_1 of the liver, blood, heart, kidney, and lungs.³³ No change was observed in the muscles or brain.

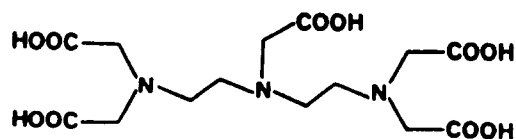
Free metal ions remain too toxic to use as safe contrast agents.³⁴ Toxicity can be lowered by rendering the ions insoluble. Colloidal manganese sulfide decreased the T_1 's of the liver and lung in rats with decreased toxicity relative to free Mn ions.³⁵ Runge et al. have

utilized suspensions of insoluble gadolinium oxalate as a gastrointestinal imaging agent.³⁶ The oxalate form is not dissociated or absorbed in the stomach; they simply pass through the intestinal tract, much like barium sulfate, and should exhibit little toxicity. Phosphate- and hydroxy-colloids of insoluble gadolinium have also been employed.³⁷ These preparations are phagocytosed by the reticuloendothelial system providing contrast enhancement of the liver and spleen, and demonstrate reduced toxicity relative to free gadolinium ions.

Another method of decreasing the toxicity of the metal ions which has received the most attention is chelation with polyaminocarboxylic acids ethylenediaminetetraacetic acid (EDTA) 3 and diethylenetriaminepentaacetic acid (DTPA) 4 with six and eight chelating sites, respectively. These ligands form strong, water soluble complexes which demonstrate remarkable in vitro and in vivo stability, a requisite for intravenous use. Cr-EDTA demonstrates potential in the assessment of renal function.³⁶



3



4

Gadolinium-DTPA is perhaps the most attractive agent at present. DTPA forms a very stable complex with Gd; the formation constant (logk) being 22.³⁸ Gadolinium has a coordination number of 9 or 10, in contrast to 6 for most transition metals. Chelation of Gadolinium with DTPA leaves at least one site open for the rapidly exchanging water

molecules. Chelation with DTPA also drastically reduces the toxicity of the gadolinium ions.

Gadolinium DTPA has been used to provide contrast enhancement of normal kidney, and to assess renal and splenic infarctions.³⁹ It has also been used in the enhancement of sterile edematous inflammatory lesions in rats³⁹ and human tumors models in mice.⁴⁰ Metal chelates normally do not cross the intact blood brain barriers. However, Gd-DTPA has been found to accumulate at focal disruptions of the blood brain barrier.³⁹ Gd-DTPA has also been used as a contrast medium in the comparison of MRI to CT in the diagnosis of cerebral tumors.⁴¹ The complex is distributed in the vascular and extracellular spaces, and thus, is a good vascular mapping agent. In addition, it has demonstrated potential as a myocardial imaging agent.^{42,43} In fact, Gd-DTPA has reached the point of clinical trials in Europe and the USA.

Current research has focused on the development of organ specific paramagnetic contrast agents. One example, is the use of liposomes as a medium for the delivery of paramagnetic species. Liposomes are man-made phospholipid vesicles consisting of lipid bilayers and inner aqueous compartments. The use of liposomes for delivery of pharmaceuticals has received much attention since Banghan et al.⁴⁴ introduced the concept of the liposome as a model system to study cell membrane transport mechanisms. The distribution of the liposome can be controlled by careful manipulation of the size, lipid composition, and polarity of the liposome. Following intravenous administration, liposomes are rapidly removed from the blood by the reticuloendothelial

system. In one case, manganese-DTPA was trapped inside the aqueous compartment of multilamellar vesicles (MLV).⁴⁵ The liposomes were smaller than 1.2 microns and selectively accumulated in the liver and spleen resulting in marked decreases in the T_1 's of those organs. Also, manganese citrate has been entrapped in liposomes.⁴⁶ Again, selective accumulation of the liposome in the liver and spleen was observed. In addition, there was significant accumulation in the brain, demonstrating the plausibility of crossing the intact blood-brain barrier by using a 30% phosphatidylserine in the liposome preparation. Hence, the use of liposomes in the development of paramagnetic contrast agents is worthy of investigation.

E. STATEMENT OF THE PROBLEM

Magnetic Resonance Imaging of the abdomen has yet to equal the quality of imaging that has been accomplished in the brain and spinal cord. Unlike the brain and spinal cord, in which there is considerable difference between proton concentration and relaxation times of the gray and white matter and cerebrospinal fluid, the tissues of the liver and spleen are quite uniform as well as similar to each other. This, in conjunction with the relative anatomical positioning of the organs of the viscera, makes tissue differentiation difficult. Paramagnetic ions entrapped in the aqueous compartment of liposomes have shown potential as T_1 enhancing agents of the liver and spleen. Liposomes are rapidly removed from the circulatory system by the reticuloendothelial system where they are endocytosed or directly taken up by the

Kupfer cells in the liver. Tissue specificity can be accomplished by manipulation of the size and lipid content of the liposomes. However, entrapment within the aqueous compartment of the liposome isolates the ions from the surrounding water of the parenchyma. As a result, the efficiency of the paramagnetic effect, which has a $1/r^6$ dependence between the resonating nucleus and paramagnetic ion, is reduced. Also, liposomes are weakened in the presence of serum albumin. As a result, the lipid bilayers become porous, and the entrapped complex leaks out and is rapidly excreted by the kidney.

The purpose of this thesis is to develop a phospholipid analog containing a paramagnetic center that can be directly incorporated into the lamellar membrane of liposomal vesicles and to investigate the efficacy of the agent as a potential MRI contrast agent of the liver, and spleen by measuring the effect of intravenous injections of this agent on the spin-lattice relaxation times of those organs in normal mice.

CHAPTER II

EXPERIMENTAL METHODS

A. GENERAL

All melting points were obtained on a Fisher-Johns Melting Point Apparatus and were uncorrected. All infrared spectra were obtained on a Perkin-Elmer 1330 Infrared Spectrophotometer and reported as cm^{-1} . Elemental analyses were provided by Galbraith Laboratories, Inc., Knoxville, Tennessee. The spin-lattice relaxation times were measured on a Joel-FX 90 Q multinuclear NMR spectrometer equipped with a 980B Texas Instrument computer. All chemicals were obtained commercially and used without further purification unless otherwise noted in Table 2. Sizing of liposomes was performed on a Hitachi H-600 Electron microscope in the University of Tennessee, Knoxville, Department of Biochemistry. Biodistribution measurements were made on 1274 BioGamma gamma counter in the Department of Biochemistry. Normal female Balb/c mice, 17-25 grams, were provided by Cumberland View Farms, Clinton, Tennessee.

B. PROCEDURES

Preparation of DTPA Dianhydride

DTPA-dianhydride was prepared by the method of Eckleman et al.⁴⁷ The anhydride was prepared several times and in different amounts. In a typical experiment DTPA (40 g, 0.102 mol) was suspended in 50 ml of pyridine in a 250 ml 3-necked flask equipped with a condenser and a

Table 2. Sources and Methods of Purification
of Commercial Chemicals

Chemicals and Abbreviations	Source ⁱ	Method of Purification ⁱⁱ
Acetic Anhydride	F	
Acetone	F	
Chloroform	F	E
Cholesterol	D	
Diethylenetriaminepentaacetic Acid (DTPA)	A	
Diethyl Ether (ether)	F	
Distilled Water (water)	B	
Egg Lecithin (Phosphatidylcholine)	C	
Ethanol (95%)	G	
Gadolinium Chloride Hexahydrate	A	
Gadolinium--153 Chloride	H	
Methanol	F	
Pyridine	A	
Stearylamine	F	

ⁱSources

- (A) = Aldrich
- (B) = Departmental still
- (C) = Avanti Polar Lipids, Inc.
- (D) = Sigma
- (F) = Fisher
- (G) = Apper
- (H) = Oak Ridge National Laboratory

ⁱⁱMethods of Purification

- (E) = Distilled from P_2O_5 and stored under N_2 atmosphere

mechanical stirrer. Acetic anhydride (38 ml, 0.402 mol) was added and the mixture stirred at 65–70°C for 24 hours which yielded a dark brown mixture. The insoluble product was collected by filtration, washed repeatedly with acetic anhydride and then ether, and dried in an oven at 50–60°C for 24 hours to give 34.19 g, 0.096 mol of a white powder (95.7%). The IR spectrum and melting point were in good agreement with literature values:³³ IR (KBr pellet, cm^{-1}) 1820, 1770, 1635; mp 180°C dec. The product was stored in a vacuum desiccator until use and did not show signs of hydrolysis after several months of storage.

Preparation of Octadecyclpentetamic Acid
Monooctadecylamminium Salt (DTPA-SA)

DTPA-SA was prepared by the method of Hnatowich et al.⁴⁸ In a typical experiment, stearylamine (1.00 g, 3.71×10^{-3} mol) was dissolved in 100 mL anhydrous chloroform in a dry 250 mL round bottom flask equipped with a reflux condensor, septum inlet, mercury bubbler, heating mantle, and magnetic stirrer, and maintained under a nitrogen atmosphere. DTPA dianhydride (1.46 g, 4.08×10^{-3} mol) was added in small lots, and then the suspension was heated at reflux for 48 hours. The insoluble product was collected by filtration and then rinsed with CHCl_3 . The remaining anhydride linkages were hydrolyzed by stirring with 100 mL of hot water for 30 minutes. The product was insoluble in water and was collected by filtration. The product was recrystallized from hot ethanol and dried in a vacuum desiccator for 24 hours to give 1.10 g, 1.86×10^{-3} mol, of a white powder (65%). The IR spectrum was

in good agreement with the literature values:³⁴ IR (KBr pellet, cm^{-1}) 2900, 1690-1720, 1640-1670; mp 110-120 dec. Elemental analysis: Calculated %C = 65.66, %H = 10.93, %N = 7.66. Found %C = 65.77, %H = 10.56, %N = 7.57.

Preparation of Gadolinium Complex of DTPA-SA (Gd-DTPA-SA)

In a typical experiment DTPA-SA (0.542 g , $5.93 \times 10^{-4} \text{ mol}$) was suspended in 50 mL of ethanol in a 100 mL round bottom flask equipped with a reflux condenser, heating mantle, and magnetic stirrer. The suspension was heated to reflux and $\text{GdCl}_3 \cdot 6\text{H}_2\text{O}$ (0.31 g , $8.34 \times 10^{-4} \text{ mol}$), dissolved in 3.0 mL of water, was added dropwise. An immediate reaction ensued resulting in a clear solution. The solution was heated at reflux for 30 min . The solution was then concentrated to $\approx 1/3$ the original volume under reduced pressure while heating; the product crystallized from the ethanol on cooling. The product was collected by filtration, rinsed with water, and then acetone, and dried in a vacuum desiccator to give 0.419 g ($3.67 \times 10^{-4} \text{ mol}$) of a fine white powder (62%): IR (KBr pellet, cm^{-1}) 2940, 1570-1630; m.p. 260°C dec.; Elemental analysis: Found: %C = 52.89, %H = 8.67 which corresponds to $\text{C}_{50}\text{H}_{97}\text{N}_5\text{O}_9\text{GdCl}_2$.

Preparation of ^{153}Gd labeled Gd-DTPA-SA

The radioisotope, ^{153}Gd , was incorporated into the Gd-DTPA-SA complex for biodistribution studies. It was prepared by a method similar to the procedure previously described. DTPA-SA (0.50 g , $5.47 \times 10^{-4} \text{ mol}$) was suspended in 50 mL of EtOH in a 100 mL round bottom flask

equipped with a reflux condensor, heating mantle, and magnetic stirrer. The ethanolic suspension was warmed to near reflux. GdCl_3 solution (3 ml, 5.74×10^{-4} mol) containing $\sim 40 \mu\text{Ci}$ of $^{153}\text{GdCl}_3$ was added slowly via a pasteur pipet. An immediate reaction ensued, and the solution turned clear; the solution was then heated at reflux for 30 minutes. The condensor was removed and replaced with a gas outlet connected to an aspirator fitted with a trap and a stopcock for pressure regulation. The solution was heated with an air gun while stirring under a slight vacuum until the volume was reduced to $\sim 1/2$ of the original volume. The hot solution was then transferred to 50 mL beaker and allowed to cool. The product crystallized, and was collected by filtration, washed with water and then acetone, and dried in a vacuum desicator to give 0.496 g, 4.35×10^{-4} mol of a white powder (79.5%).

Preparation of Gadolinium Labeled Liposomes (GLL)

Numerous preparations of liposomal suspensions were made in which the composition of Gd-DTPA-SA was varied from 16 to 53 mole percent, and the total lipid concentration varied from 17 to 95 $\mu\text{mol/ml}$. Representative preparations are summarized in Table 3. The liposomes were prepared by adding the appropriate amount of Gd-DTPA-SA in methanol to an equimolar mixture of egg lecithin and cholesterol in chloroform.⁴⁹ Preparation number 7 in Table 3 is a typical example. Thirty μmol of Gd-DTPA-SA in methanol was added to 30 μmol of egg lecithin and 30 μmol of cholesterol in chloroform. The solvent was removed by a stream of

Table 3. Summary of the Preparation of Liposomes Involving Various Lipid Concentrations

Preparation Number	Mole % Gd-DTPA-SA	Total Lipid Concentration ($\mu\text{mol/ml}$)	Quantities of Liposomal Constituents (μmoles)		
			Gd-DTPA-SA	Lecithin	Cholesterol
1	16	19	3	8	8
2	16	95	15	40	40
3	33	18	6	6	6
4	33	30	10	10	10
5	33	36	12	12	12
6	33	54	18	18	18
7	33	90	30	30	30
8	53	17	9	4	4
9	53	43	23	10	10
10	53	85	45	20	20

N_2 followed by vacuum desiccation. The dried mixture was resuspended in 1.0 ml of phosphate buffered saline (PBS), pH 7.4, to give a total lipid concentration of 90 $\mu\text{mol/ml}$. The suspension was then either briefly sonicated in a bath sonicator to form large multilamellar vesicles (MLV) or sonicated for up to two hours to produce small unilamellar vesicles (SUV).

Radioactive liposomes were prepared via the same procedure by the addition of the appropriate amount of $^{153}\text{Gd-DTPA-SA}$ preparation.

The liposomes were sized by electron microscopy.

Animal Protocol and Sample Preparation

Normal female Balb/c mice, 17-25 g, were given dosages ranging from 2.0 to 18.0 μmol per animal of the GLL preparations via the tail vein. Also, a series of mice received injections of either PBS or "empty" liposomes as controls. The volume injected never exceeded 200 μl . The animals were sacrificed by cervical dislocation at periods ranging from 5 minutes to several months postinjection. The liver and spleen were removed and immediately placed in separate glass test tubes covered with parafilm and placed in ice until the relaxation time was measured. The T_1 's of the tissues were measured within 1 hour post-mortem at $4 \pm 2^\circ\text{C}$ unless otherwise noted.⁵⁰ The liver and lung tissues were very efficiently aspirated to the bottom of 5 mm NMR tubes with a 12" long 18 gauge stainless steel needle with minimal tissue damage. Due to the nature of the tissue, the spleens had to be pushed part of the way down the NMR tubes with a glass rod, then they were easily

shaken to the bottom. The process of packing the tubes took no longer than 2 minutes per sample.

Spin-Lattice Relaxation Time Measurement

The T_1 was measured at 4°C unless otherwise noted using a 90 MHz, JOEL FX-90Q multinuclear NMR spectrometer operating at 89.55 MHz and a static field strength of 2.1 Tesla. The T_1 was determined using an inversion recovery sequence (see Appendix). PD was set to an interval greater than five times T_1 . Seven values of PI were used. The PI's increased stepwise resulting in a curve of magnetization (M_z) recovery time similar to that shown on page 9. T_1 was computed by the computer from a least squares fit of the acquired curve. The correlation coefficient of the fitted curves was > 0.999 for all samples.

Biodistribution Studies

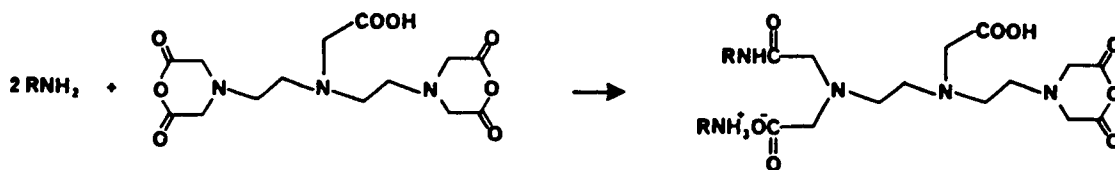
In the biodistribution studies normal female Balb/c mice, 17-25 g, received 9 μmol of the 33.3 mole % $^{153}\text{Gd-DTPA-SA}$ SUV liposome preparation. The mice were sacrificed from 30 minutes to 30 days later, and a sample of blood, the liver, lungs, heart, stomach, kidneys, and spleen were removed, weighed, and placed in a gamma counter (Beckman) to measure the percent of radioactivity on a per organ and per gram basis. At least three mice were used per time period.

CHAPTER III

RESULTS AND DISCUSSION

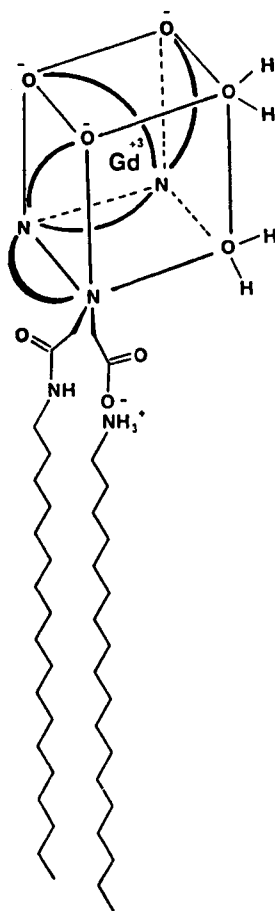
A. PREPARATION OF THE Gd-DTPA-SA COMPLEX

The preparation of the lipophilic gadolinium complex from a stearylamine diethylenetriaminepentaacetic acid is complicated by the fact that the multifunctional DTPA portion of the molecule permits a number of isomers to be formed. The stearylamine linkage to the DTPA is almost certainly at one of the terminal acetic acid groups due to the method of formation of the linkage. That is, stearylamine would be expected to react much more rapidly with an anhydride linkage than with a free acid. The presence of a certain percentage of the complex in which the stearylamine is bonded to the central acid moiety cannot be ruled out in this initial investigation. A further complication



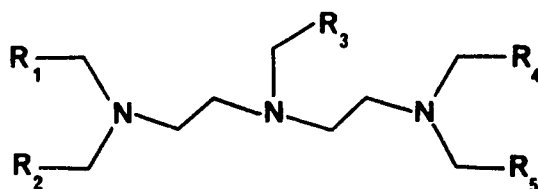
involves the fact that elemental analysis reveals that the stearylamine-DTPA complexing agent is actually a stearylaminium salt. Since the salt linkages are labile in solution, it must be assumed that the resultant gadolinium complexes are actually mixtures of isomeric salts. The potential isomeric DTPA-stearylamine salts are summarized

in Table 4. A plausible structure for the gadolinium complex of isomer number 1 of Table 4 is represented below. Notice that the complex resembles a phospholipid with a polar, paramagnetic head and a nonpolar hydrocarbon tail.



The elemental analysis reported in the experimental section reveals that a normal preparation of the complex contains two chloride ions. We have observed variations of this formula presumably due to variations in the coordination parameters during sequential preparations. Since the preparations are adjusted to neutral pH via the addition of phosphate buffers during liposome formation, relatively little is known of the actual coordination number of the gadolinium in

Table 4. Potential Isomers of the DTPA-SA Ligand



Isomer Number	R ₁	R ₂	R ₃	R ₄	R ₅
1	a	b	c	c	c
2	a	c	b	c	c
3	a	c	c	b	c
4	a	c	c	c	b

a = $-\text{HN}(\text{CH}_2)_{17}\text{CH}_3$

b = $-\text{O}^-\text{NH}_3^+(\text{CH}_2)_{17}\text{CH}_3$

c = $-\text{OH}$

the final complex. Based on earlier work utilizing simple Gd-DTPA complexes, it is assumed to be between eight and ten.

B. PREPARATION OF THE GADOLINIUM LABELED LIPOSOMES

The paramagnetic liposomes were prepared by incorporating the paramagnetic phospholipid analog in the lamellar membrane of liposomes as illustrated in Figure 5. In tracer studies using Gd-153, it was found that approximately 80% of the added Gd-DTPA-SA was incorporated into the 33.3 mole% SUVs as determined by size exclusion chromatography (Sephacrose CL-6B beads, Pharmacia Fine Chemicals) of the Gd-153 labeled liposomes. In addition, the percent activity of the ¹⁵³Gd-DTPA-SA labeled liposomes remained constant up to 20 days, indicating good in vitro stability of both the liposomes and the Gd-DTPA-SA complex.

The average size of the gadolinium labeled MLVs and SUVs were 0.12 μ m and 0.04 μ m, respectively, compared to 0.22 μ m and 0.07 μ m, respectively, for control liposomes containing only equimolar quantities of cholesterol and phosphatidylcholine. The size distributions of the liposomes are summarized in Figures 6 and 7. The T_1 of a 53 mole%, 85 μ m/ml MLV preparation was measured to be 33.0 ms at 4°C compared to 1.48 sec for the same concentration of a control MLV suspension.

C. EFFECTS OF I.V. ADMINISTRATION OF THE GLL ON THE SPIN-LATTICE RELAXATION TIMES OF THE LIVER AND SPLEEN

In the initial studies, each mouse received 3 μ moles of a MLV preparation containing varying mole percentages of the Gd-DTPA-SA

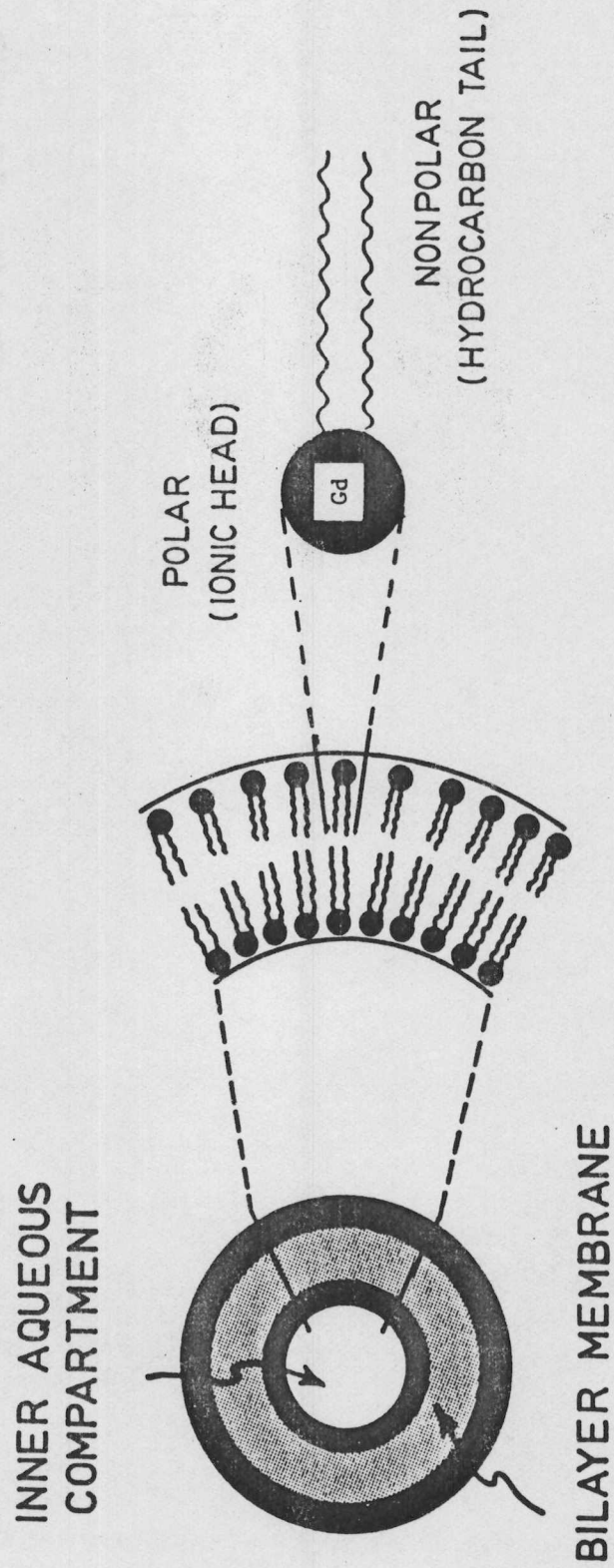


Figure 5. Formation of a paramagnetic liposome.

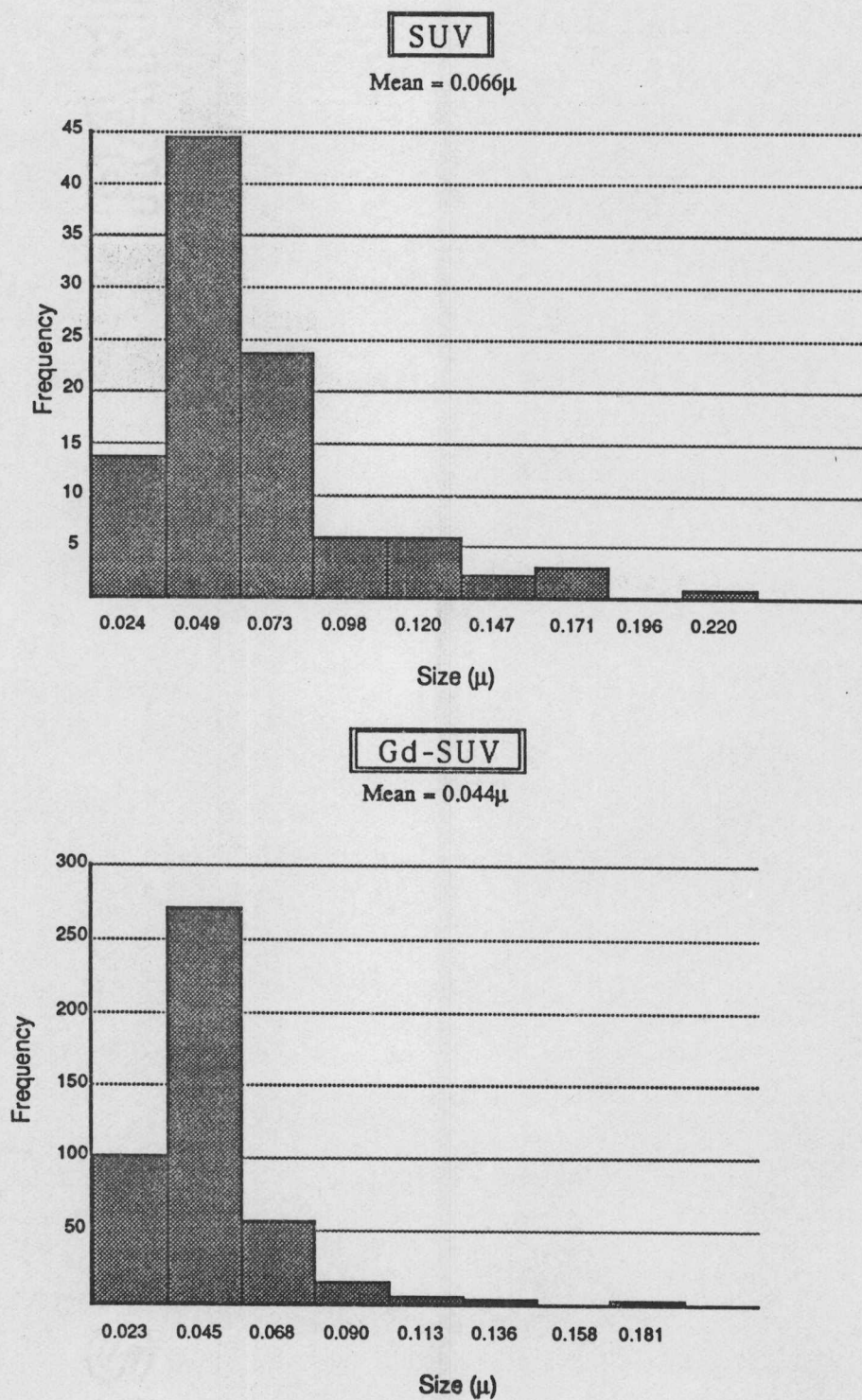


Figure 6. Size distribution of the SUVs and Gd-SUVs.

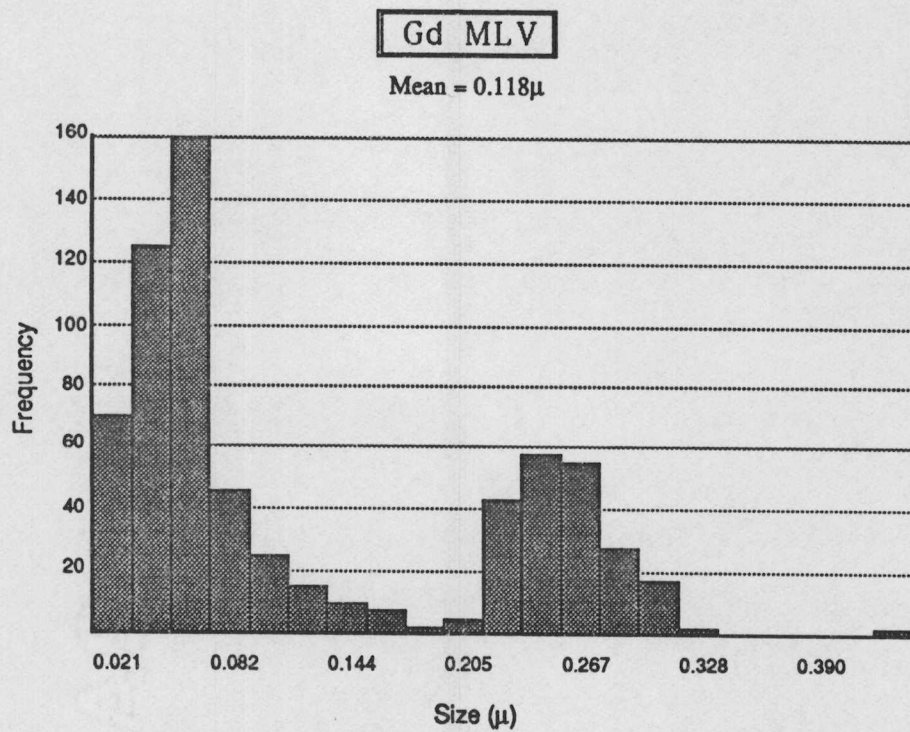
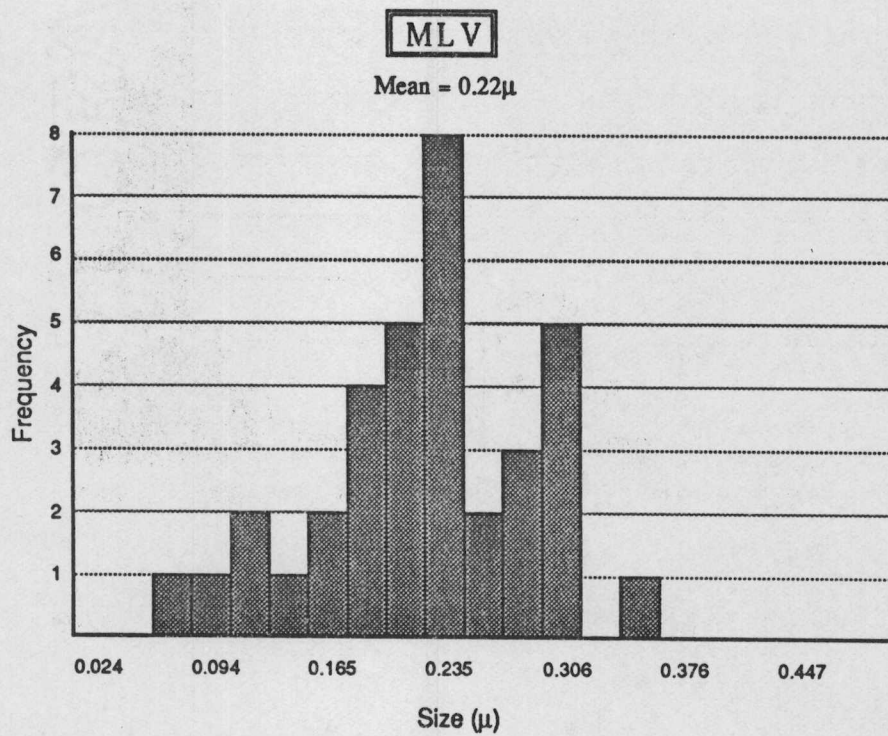
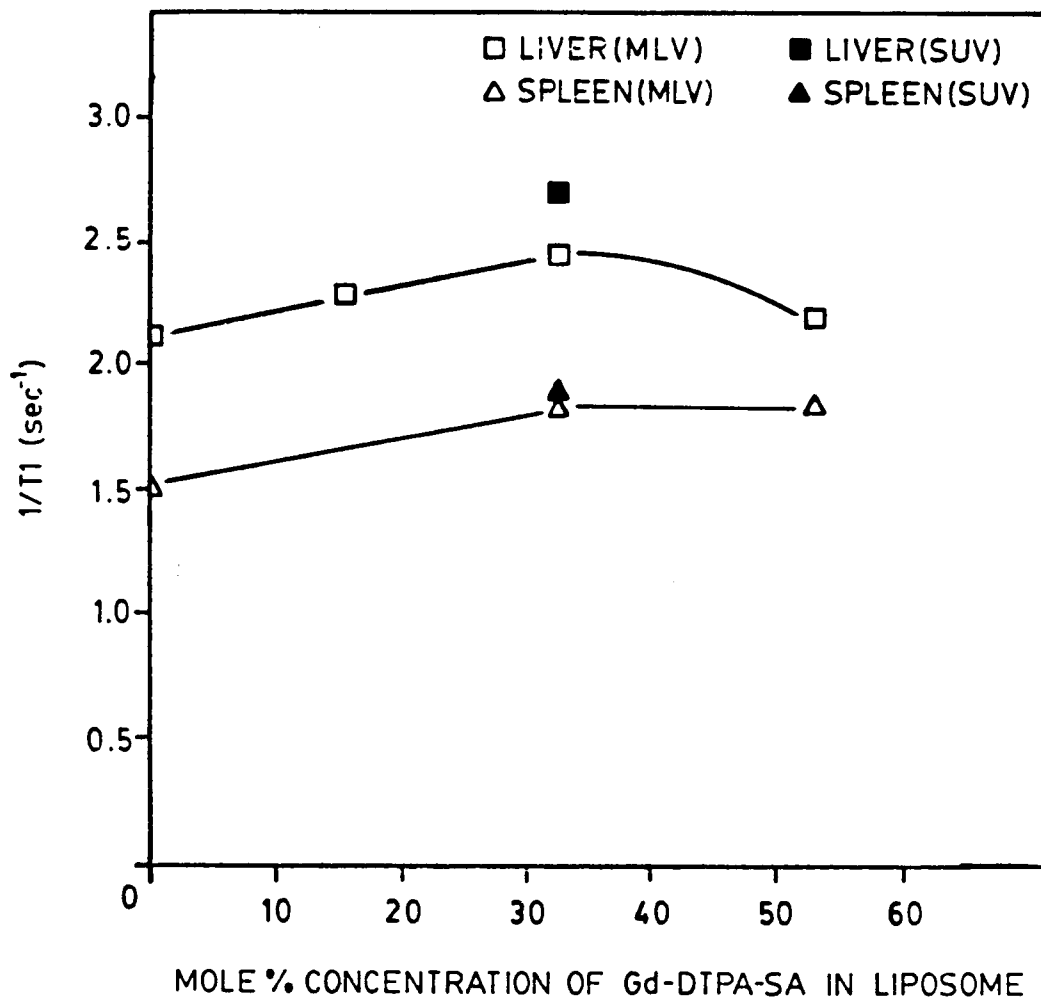


Figure 7. Size distribution of the MLVs and Gd-MLVs.

complex. The results of these experiments are summarized in Figure 8. Interestingly, the MLV preparation actually contains a significant amount of SUVs. The 33.3 mole% Gd-DTPA-SA preparation was found to give the maximum increases in the relaxation rates in which relaxation rates of the liver increased 14% and the spleen by 27% compared to controls. The observed effect was considered to be real since the percent increase was greater than the percent standard deviation from the mean of control values. An additional experiment was carried out to study the dose effect of the 33.3 mole% MLV preparation on the relaxation rate of the liver and spleen. The mice received dosages ranging from 3.0 to 9.0 μ moles of lipid/mouse, which corresponds to 0.047 to 0.143 mmoles of gadolinium per kilogram of body weight. The results are summarized in Figure 9 and Table 5. The response was linear over the range of dose given. The slope of the regression line for the liver response was 33% greater than that for the spleen, indicating that the agent was somewhat more selective for the liver. The relaxation rate of the liver was increased by 48% compared to 31% for the spleen at a dose of 9.0 μ moles of lipid/mouse.

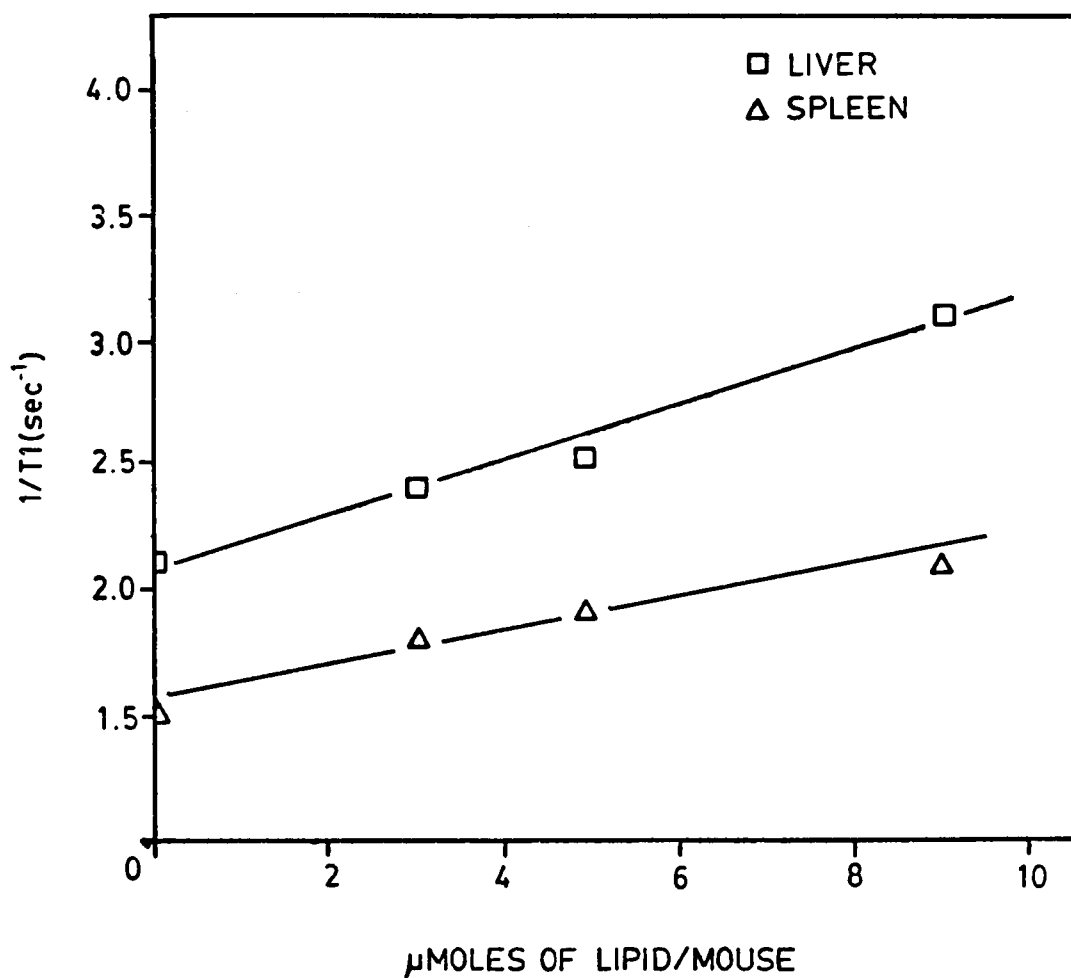
It is well established that the distribution of the liposomes can be controlled by careful manipulation of the size of the vesicles. Smaller liposomes should be more selective than larger ones. It has been shown that larger liposomes become trapped in the capillaries due to their physical size, where as smaller liposomes remain in the blood stream longer allowing for selective tissue discrimination. In addition the size distribution of the Gd-MLV preparations were bimodal.



mole% Gd-DTPA-SA	Liver		Spleen	
	N	T ₁ ave*	N	T ₁ ave*
Control	11	472 ± 6%	7	657 ± 4%
16	2	443 ± 6%	-	-
33	3(2)	421 ± 4% (367 ± 3%)	2(2)	544 ± 4% (536 ± 3%)
53	2	454 ± 4%±	2	568 ± 5%

*MLV(SUV).

Figure 8. Effect of mole% Gd-DTPA-SA in SUV and MLV preparations on T₁ of the liver and spleen (dose 3 μmole lipid/mouse).



<u>Organ</u>	<u>Slope*</u>	<u>Correlation coefficient</u>
Liver	2.06	0.98
Spleen	1.55	0.98

*Computed from the linear regression analysis of the acquired data on a TI-55 III Texas Instrument hand calculator.

Figure 9. Effect of quantity of 33.3 mole% Gd-DTPA-SA MLV liposome on relaxation rates of the liver and spleen.

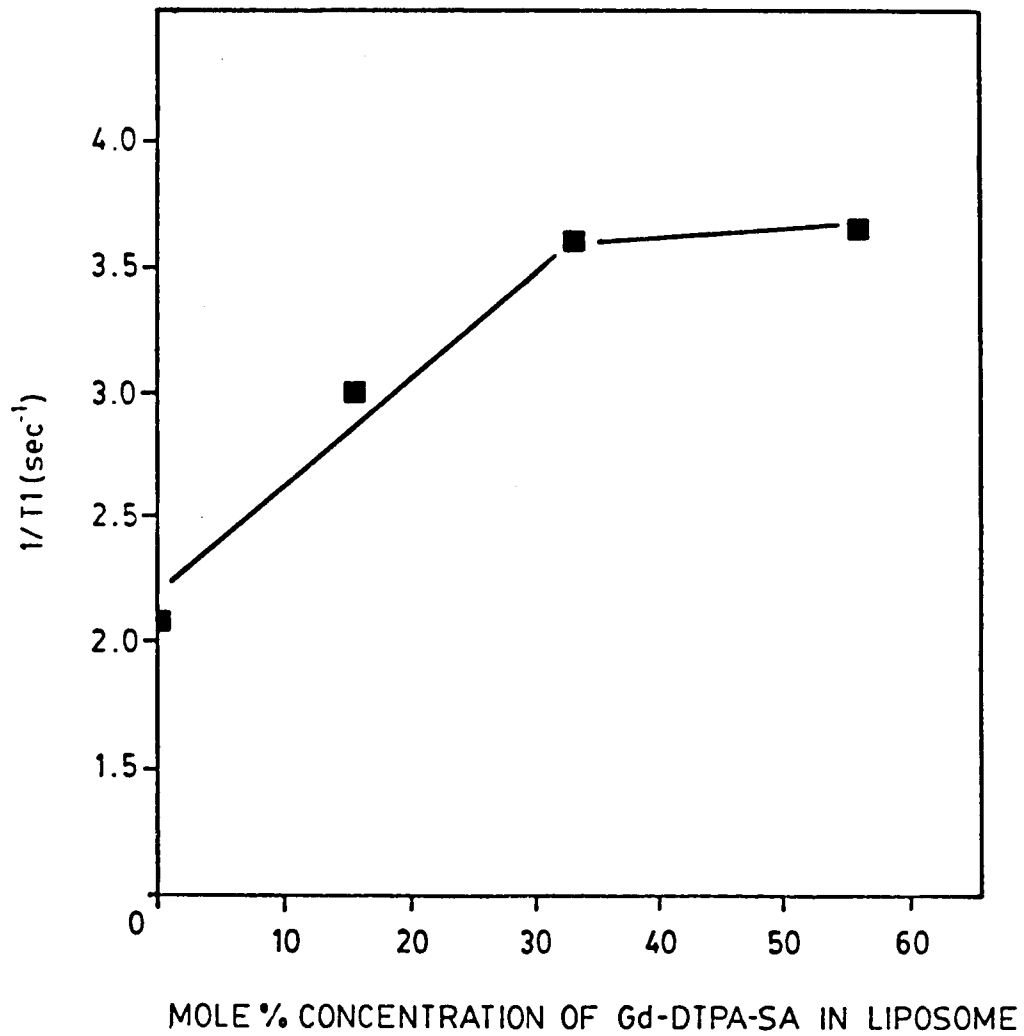
Table 5. Dose Effect of 33.3 Mole% Gd-DTPA-SA MLV

Dose ($\mu\text{mol}/\text{mouse}$)	Liver		Spleen	
	N	T_1 ave	N	T_1 ave
control	11	$472 \pm 6\%$	7	$657 \pm 4\%$
3	3	$421 \pm 4\%$	3	$544 \pm 6\%$
5	2	$403 \pm 5\%$	2	$519 \pm 4\%$
9	16	$327 \pm 11\%$	6	$485 \pm 5\%$

Therefore, a study was carried out to investigate the use of SUVs as opposed to MLVs for further enhancement of the liver and spleen relaxation times. Each mouse received 3.0 μ moles of a 33.3 mole% Gd-DTPA-SA preparation of SUVs. These results are also summarized in Figure 8. The SUVs caused an increase of 29% in the relaxation rate of the liver as opposed to a 14% increase caused by the MLVs. However, the relaxation rate of the spleen remained essentially unchanged compared to the MLVs, demonstrating increased tissue selectivity.

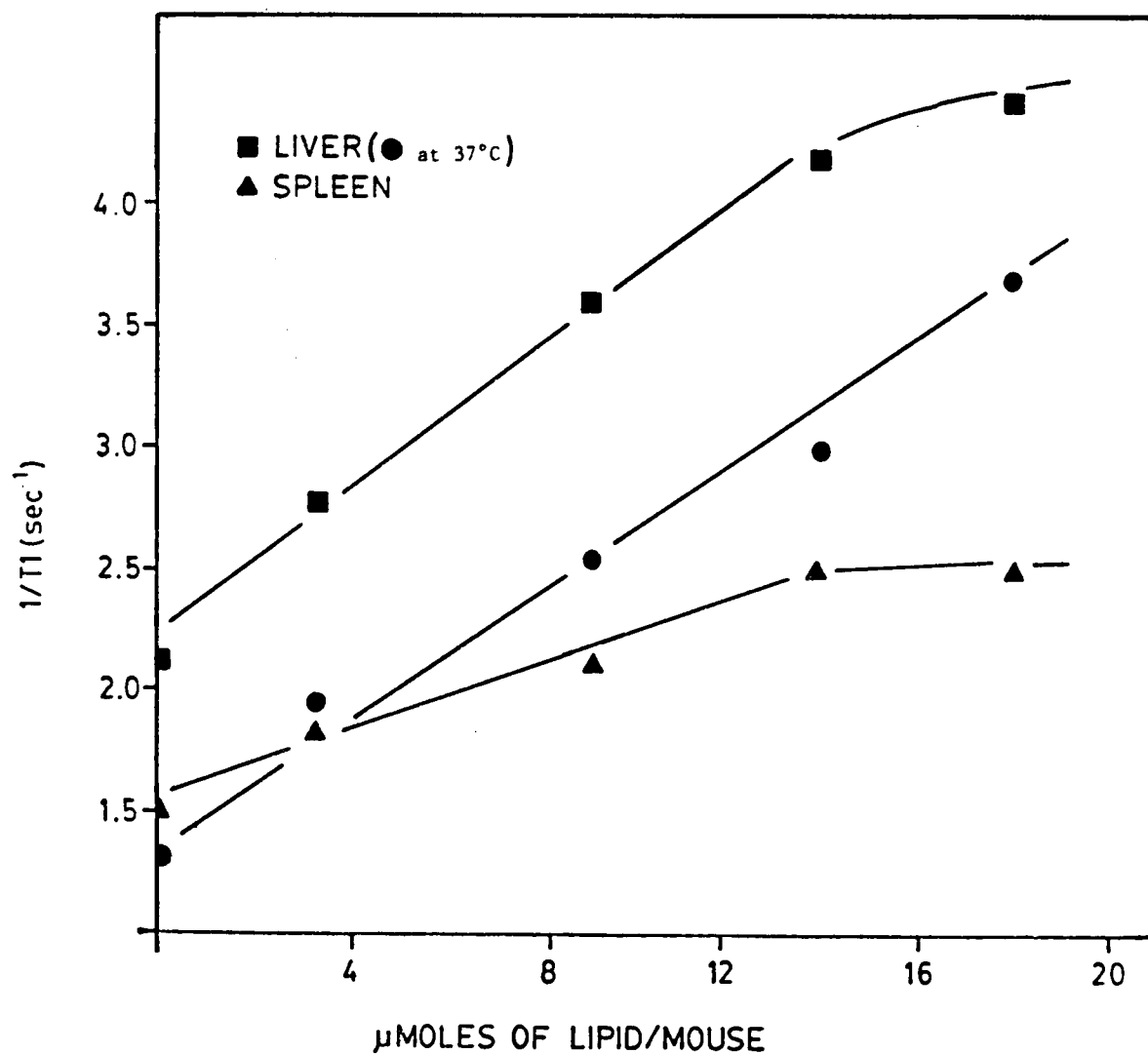
Since the SUVs were more effective at reducing the T_1 of the liver, a study was carried out in which the mole percent of Gd-DTPA-SA was varied. The mice received 9.0 μ moles of lipid per mouse. The results are summarized in Figure 10. As with the MLVs, the maximum increase in relaxation rate of the liver was observed at approximately 33.3 mole% Gd-DTPA-SA. The relaxation rate was increased by 71%.

To better evaluate the 33.3 mole% SUV preparation as a contrast medium, a dose-response study was carried out in which mice received dosages ranging from 3.6 to 18 μ moles per mouse of the 33.3 mole% preparation, and the relaxation times of the liver, spleen and lung were measured. The results are summarized in Figure 11 and Table 6. The response varied linearly up to 14 μ moles of lipid and then appeared to level off. The slope of the regression line for the liver response, up to 14 μ moles, was 45% greater than that for the spleen, compared to a 33% enhancement caused by the MLVs. This corresponds to a 12% increase in selectivity of the liver for the SUVs. At a dose of 14 μ moles of lipid per mouse, the relaxation rate of the liver and



<u>Mole%</u> <u>Gd-DTPA-SA</u>	<u>N</u>	<u>T₁ ave</u>
control	11	472 ± 6%
16	4	332 ± 7%
33	15	279 ± 13%
53	4	269 ± 6%

Figure 10. Effect of mole% Gd-DTPA-SA in SUV liposomes on T_1 of the liver.



<u>Organ</u>	<u>Slope</u>	<u>Correlation coefficient</u>
Liver	2.19	1.0
Spleen	1.51	1.0

Figure 11. Dose-response of 33.3 mole% Gd-DTPA-SA SUV liposome on relaxation rates of the liver and spleen.

Table 6. Dose Effect of 33.3 Mole% Gd-DTPA-SA SUV

Dose ($\mu\text{mol}/\text{mouse}$)	Liver		Liver (37°C)		Spleen	
	N	T_1 ave	N	T_1 ave	N	T_1 ave
control	11	$472 \pm 6\%$	4	$766 \pm 7\%$	7	$657 \pm 4\%$
3.6	2	$362 \pm 2\%$	4	$517 \pm 11\%$	2	$453 \pm 6\%$
9.0	15	$279 \pm 13\%$	4	$388 \pm 8\%$	4	$475 \pm 2\%$
14	2	$237 \pm 7\%$	4	$331 \pm 7\%$	3	$394 \pm 4\%$
18	2	$228 \pm 2\%$	4	$269 \pm 23\%$	3	$398 \pm 5\%$

spleen increased by 100 and 67%, respectively. The dose that achieved these results corresponds to a dose of 0.22 mmoles of gadolinium/kg of body weight, which compares favorably with a dose of 0.10 to 0.2 mmoles of Gd-DTPA/kg currently used in human imaging of the central nervous system.

The leveling off of the dose effect at 14 μ moles may be due to saturation of the tissue with the liposomes.⁴⁵ For example, one strategy for controlling the distribution of liposomes is to preadminister large doses of empty liposomes which accumulate in the liver and spleen and thus divert the uptake of the loaded liposomes from the hepato-splenic axis. This effect may be what is observed when more than 14 μ moles are injected.

The dose effect was also measured at 37°C for the liver in an attempt to mimic in vivo conditions. The results are also plotted in Figure 11. Since the relaxation rates are inversely related to

$$\frac{1}{T_1} = \frac{12\pi^2 \gamma^2 \mu_{\text{eff}}^2 \eta N_p}{5kT}$$

temperature, it was not surprising, to find that the relaxation rates were decreased relative to those measured at 4°C. However, the correlation of relaxation rates with administered dosage parallels that obtained at 4°C. The data is more difficult to interpret due to the degeneration of the tissue.³⁴ At 37°C the decay of the tissue is more pronounced and as the tissue decays the relaxation times become shorter. Also, the rate of decay is different for each animal which

affects the reproducibility of the experimental data leading to larger standard deviation. In some instances the T_1 s varied as much as 20%.

Since large differences in relaxation rates lead to enhanced MRI contrast, one would expect that images of the liver and spleen generated 30 min post I.V. administration of 0.67 μ moles/kg of the 33.3 mole% GD-DTPA-SA SUV preparation should demonstrate good contrast enhancement.

D. BIODISTRIBUTION STUDIES

Biodistribution studies were carried out to correlate the observed effect of the GLLs on the various organs. These results are summarized in Figures 12 and 13 and Table 7. As expected, the SUVs were selective for the liver and spleen. At 30 min, 44% of the liposome had accumulated in the liver and 12% in the spleen, while 35% remained in the circulatory system. Only a 2% accumulation was observed in the lungs. T_1 measurements of the lungs at 30 min postinjection indicated a 100% increase in relaxation rate at a dose of 14 μ moles of lipid/mouse. This effect is thought to be caused by the large perfusion of blood within the lung tissue. Within 24 hr the quantity of liposome in the blood had decreased to 4%. The accumulation within the liver reached 70% in 24 hrs compared to 21% for the spleen. During a one month period, the activity in the kidneys never exceeded 1.6% indicating good in vivo stability of the Gd-DTPA-SA complex. If the complex were degraded and Gd-DTPA expelled, one would expect to see a rise in the activity in the kidneys since Gd-DTPA is rapidly removed by the renal system.

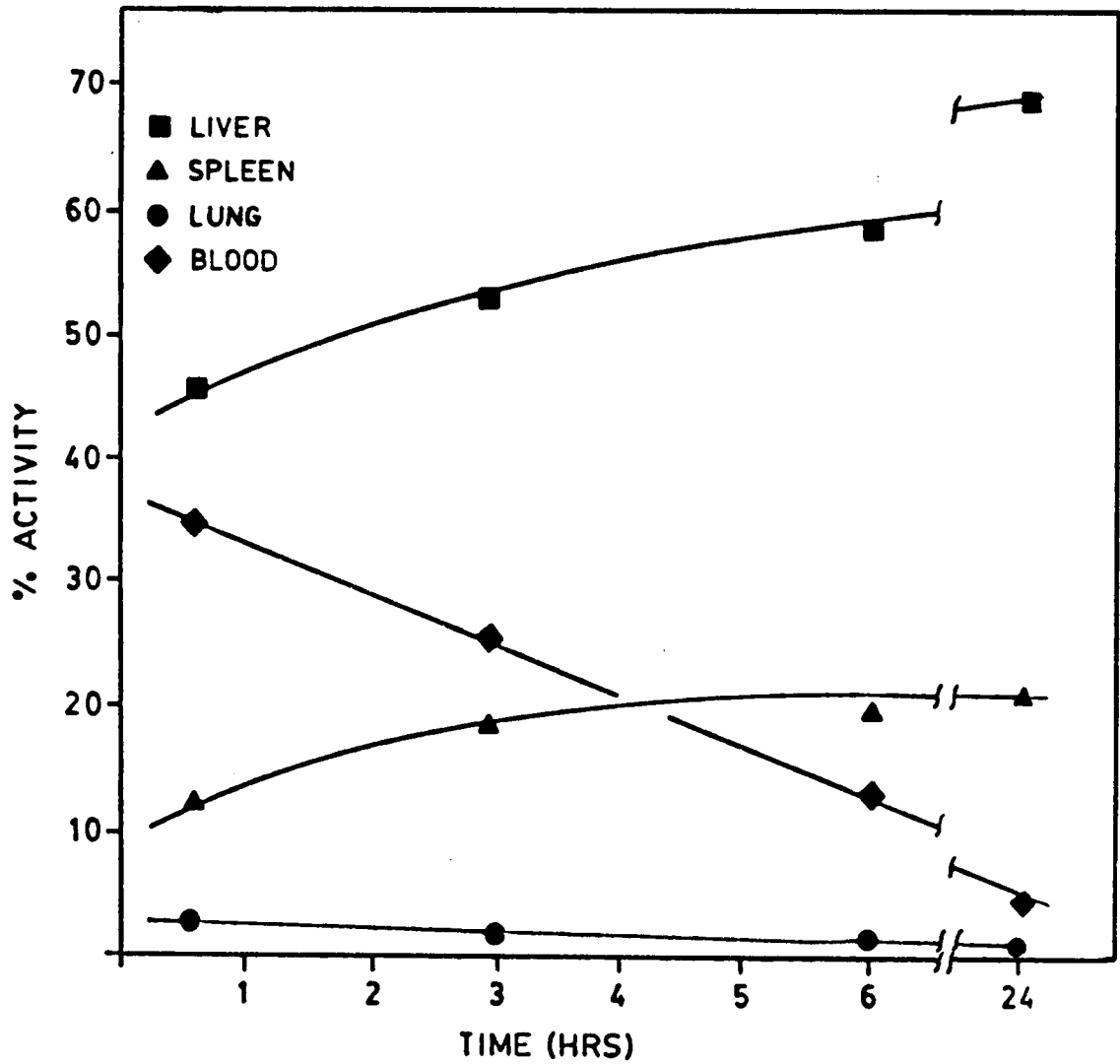


Figure 12. Distribution of $^{153}\text{Gd-III}$ per organ. (9 μmoles of SUV lipid/mouse; 33.3 mole% $^{153}\text{Gd-DTPA-SA}$.)

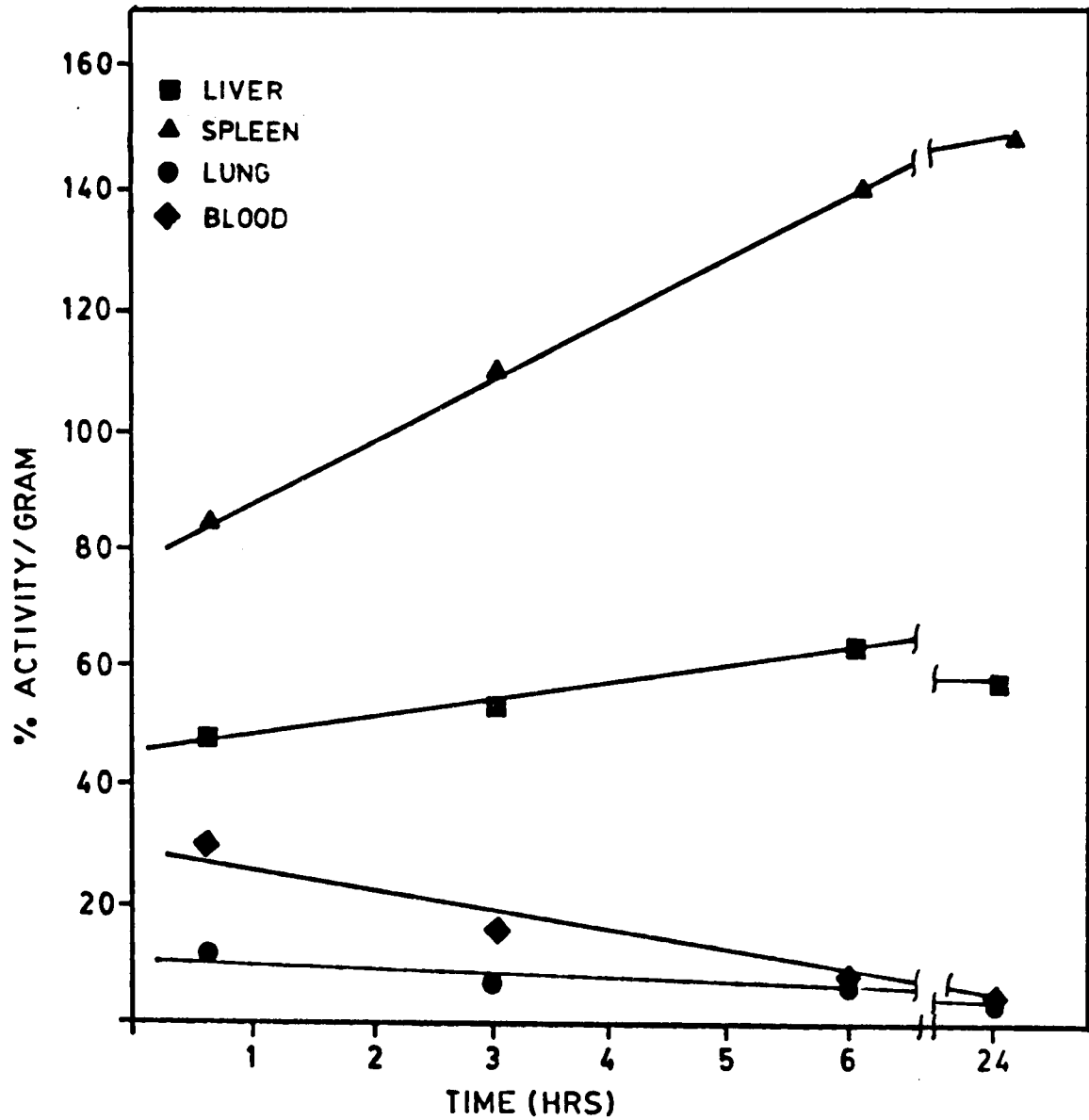


Figure 13. Distribution of $^{153}\text{Gd-LL}$ per gram of tissue. (9 μmoles of SUV liposome; 33.3 mole% $^{153}\text{Gd-DTPA-SA}$.)

Table 7. Percentage of Activity per Organ of $^{153}\text{Gd-LL}$
 (9 $\mu\text{moles lipid/mouse}$ of 33.3 mole% $^{153}\text{Gd-DTPA-SA SUV}$)

Organ	Time						
	Hours				Days		
	0.5	3	6	24	2	11	30
Liver	44	53	58	70	84	83	81
Blood	35	24	15	4	0.2	0.2	-
Spleen	12	17	20	21	11	13	13
Lung	2	1	1	0.4	0.1	0.3	0.2
Kidney	1	0.8	1.6	0.9	1.0	1.0	0.7
Heart	1	0.6	0.6	0.2	0.9	0.1	0.02
Stomach	0.7	0.5	1	0.6	0.6	0.5	2.8

One major disadvantage of this agent is its very long retention time. After one month at least 95% of the administered dose remains in vivo, with 81% in the liver. Ideally an image contrast agent will remain only long enough to allow data acquisition. The retention time of this agent may possibly be reduced by the introduction of a biodegradable aliphatic side chain. It is important to note that there have been no acute toxicities of the agent observed. In fact, of the studies made, an LD₅₀ has not been determined because none of the mice died from the agent during the inoculation period.

E. CONCLUSION

Covalently linking Gd-DTPA to stearylamine resulted in the formation of a lipophilic complex that was effectively incorporated in the lamellar membrane of liposomal vesicles. Intravenous injections of the paramagnetic liposomes reduced the spin-lattice relaxation times of the liver and spleen by as much as 100 and 67%, respectively. In this initial study no acute toxicities have been observed. However, due to the prolonged retention time of the agent in the liver, further studies of the chemical, physical, and pharmacokinetic properties are suggested.

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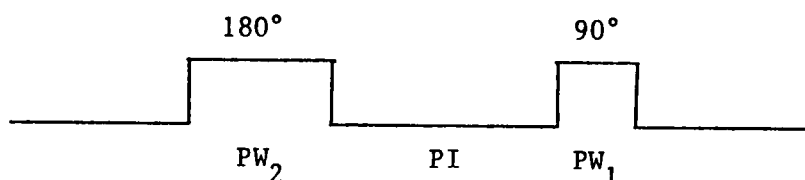
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APPENDIX

Procedure for Measuring the Spin-Lattice Relaxation

Time Using the Inversion Recovery Pulse Sequence

First, in the single pulse mode, PW_1 was carefully adjusted until a nulled spectrum was obtained. The value of PW_1 which gave the nulled spectrum is the 180° pulse. The 90° pulse = 180° pulse/2. Second, in the double pulse mode, PW_1 was assigned the 90° value, and PW_2 was assigned the 180° value as described below.



While in the double pulse mode, PI_{null} was determined by adjusting PI until a nulled spectrum was obtained. Seven PI values were then determined, six ranging from $0.5 \times PI_{null}$ to $1.5 \times PI_{null}$ and one at $5 \times PI_{null}$. PD was set at $5 \times PI_{null}$. The PI values were entered in the stack program such that data #1 received the $5 \times PI_{null}$ value and data #2 through #7 received values increasing from 0.5 to $1.5 \times PI_{null}$ respectively in increments of $(1.5 \times PI_{null} - 0.5 \times PI_{null}) \div 5$. The results are plotted, and the T_1 is calculated by the computer from the slope of the line generated from the graph of peak intensity vs. PI . Using this procedure the T_1 of water at $20^\circ C$ was determined to be 2.4 ± 0.1 , compared to 2.3 ± 0.5 obtained by Bloembergen et al.⁷ An example of a series of spectra from a typical T_1 measurement is illustrated in Figure 14.

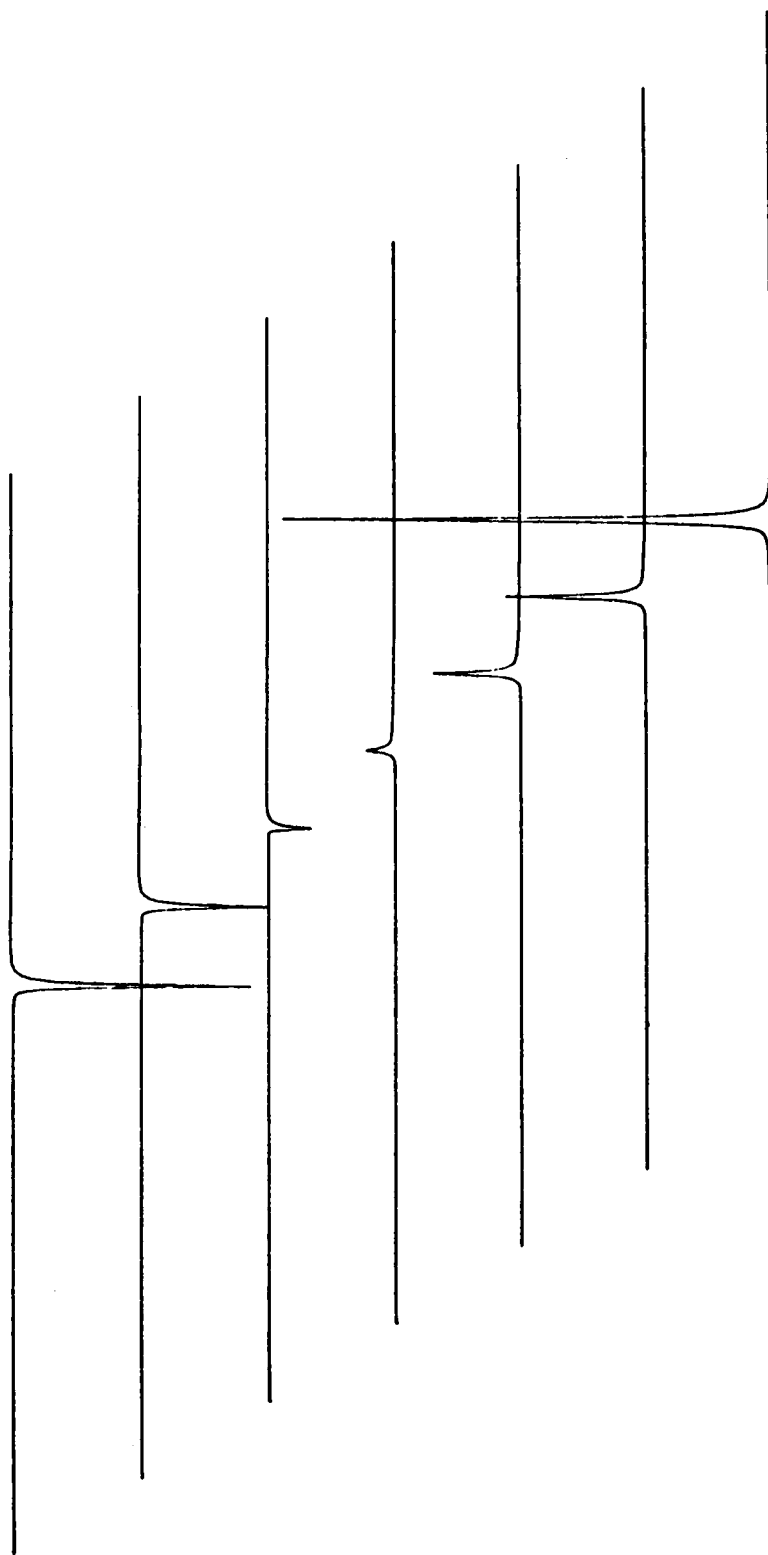


Figure 14. Typical spectra of a T_1 measurement of water.

VITA

Thomas Hudson Moss, III was born in Rome, Georgia, where he graduated from Darlington High School in 1974. While working part-time, he attended Shorter College in Rome where he received his Bachelor of Science degree in chemistry in May 1982. He then worked for one year before accepting a Graduate Teaching Assistantship at the University of Tennessee, Knoxville, in August 1983.

The author is presently employed by Southern Research Institute in Birmingham, Alabama.