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Intravascular and Intracellular Hepatic Relaxivities of Superparamagnetic Particles: An Isolated and Perfused Organ Pharmacokinetics Study

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The relative contributions of intravascular and intracellular compartments to the proton transverse relaxation of the isolated and excised rat liver were determined during the phagocytosis of superparamagnetic particles. The evolution of the proton transverse magnetization of the organ perfused with increasing doses of starch-coated magnetic microspheres was followed up using a Carr–Purcell–Meiboom–Gill sequence with various echo times. From the multiexponential fit of the echo train, the amplitudes and the relaxation rates R_2 of the liver tissue were obtained. The results clearly indicate that shortly after contrast medium administration, an internalization takes place which can be followed by the rapid and biphasic evolution of the transverse relaxation rate of the water protons. A very fast decaying component looking like an initial loss of the magnetization is observed together with an increase of the relaxation rate of the remaining water tissue. This regime is strongly dependent on both the echo time and the iron concentration, a behavior characteristic of the agglomeration of magnetic particles. The examination of the liver tissues by electron microscopy shows that this clustering arises in cytoplasmic vacuoles.

Key Words: **MRI contrast agents; superparamagnetic particles; relaxometry; perfused liver.**

INTRODUCTION

In 1984, iron oxide particles were proposed by Olsson *et al.* (*1*) as MRI contrast agents for both the liver and the spleen. Their synthesis is usually based on the precipitation of iron oxide in an alkaline solution containing a coating agent such as polysaccharides to prevent the agglomeration of the magnetic cores constituting the active part of these materials. After intravenous administration, these agents, now called SPIO (superparamagnetic iron oxides) or USPIO (ultrasmall superparamagnetic iron oxides), are mainly cleared from the blood by Kupffer cells, the fixed macrophages of the liver (*2*).

Owing to their large magnetic moment, superparamagnetic particles create an inhomogeneous microscopic distribution of the magnetic field around the Kupffer cells which accelerates the dephasing of the spins—and hence the R_2—of water protons and consequently induces an attenuation of the signal intensity of the targeted area (*3, 4*). In contrast, and owing to a low population of macrophages, liver tumors are unable to sequester detectable amounts of SPIO. As a result, the contrast between normal liver tissue and tumors is greatly enhanced (*5*).

In the presence of well-dispersed magnetic crystals, the evolution of the transverse magnetization of water protons recorded with a Carr–Purcell–Meiboom–Gill sequence is perfectly monoexponential and totally independent of the echo time (TE) as long as it is longer than the time required for water molecules to diffuse from one particle to another one (*6*). By comparison, large agglomerates induce a very fast decay of the transverse magnetization, which is attributable to the protons close to the magnetic centers and whose spins are totally and irreversibly dephased within the first refocusing TE period. The amplitude of this very short decay increases with both the iron concentration and the TE length (*6*). Because of the coexistence of several populations of water molecules and the time scale of the exchange between them, the regime of the decay of the transverse relaxation becomes multiexponential. As stressed earlier, this information is of paramount importance in the context of the biological characterization of particulate contrast materials (*6*). A monoexponential behavior is indeed expected if the particles remain dispersed after their intravenous administration; but any aggregation occuring during the clearance process will induce a multiexponential behavior likely to be characterized by a very fast decaying component looking like an initial loss (IL) of the magnetization.

In an earlier study performed on the isolated rat liver perfused with a solution free of proteins (*7*), we demonstrated that magnetic starch microspheres (MSM), a superparamagnetic contrast agent made of iron oxide cores of a diameter of about 10 nm dispersed in starch, are rapidly cleared up by the Kupffer cells through a lectin-mediated endocytosis process. The overall size of the microspheres is about 200 nm. In the presence of rat blood, the particles are opsonized with immunoglobulins which are recognized by specific receptors at the surface of Kupffer cells (*8*). In both situations, the uptake process involves (i) the attachment of the particles to receptors located on the cytoplasmic membrane, (ii) the subsequent internalization and agglomeration of the complex in digestive vacuoles, and (iii) the dissolution of the ingested material. In

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the context of MRI, these sequential events could diversely affect the contrast induced by the particulate contrast agent.

The present work aims to study the various internalization steps through the analysis of the multiexponential behavior of the transverse magnetization of water protons in isolated rat liver tissue perfused with MSM.

MATERIALS AND METHODS

In Vitro Characterization of MSM

The transverse relaxivity r_2 of MSM was determined in the Krebs–Henseleit bicarbonate buffer (K-H) used as perfusion fluid for the liver. The composition of this fluid (in millimolar) is as follows: NaCl 118, KCl 4.7, $CaCl_2$ 3, $MgSO_4$ 1.2, Na_2H_2EDTA 0.5, $NaHCO_3$ 25, D-glucose 5.5, and pyruvic acid 5.5.

For relaxivity determinations, the transverse relaxation rates R_2 of K-H solutions containing increasing concentrations of MSM were measured at 37°C on a spin analyzer Minispec PC-20 (Bruker, Karlsruhe, Germany) working at 0.47 T. The measurements were performed with a Carr–Purcell–Meiboom–Gill (CPMG) sequence for two different echo times (TE), 0.4 and 4 ms. Data collection included the acquisition of the amplitude of the free induction decay (FID) observed 20 μs after the first 90° pulse. The experimental data of the transverse magnetization decay curves were processed on a personal computer, with a multiparametric minimization program (Minuit, CERN Library Program) (*9*). This treatment identifies the mono- or multiexponential character of the decay and gives the value(s) of R_2 and, if any, the initial loss of the magnetization. This IL accounts for any mechanism occurring during the first TE period; its value corresponds to the difference between the amplitude of the magnetization extrapolated to the origin and the value acquired 30 μs after the initial 90° pulse.

For the iron concentration measurement, the solution was brought at pH < 1 by addition of a 10% aqueous HCl and kept in a hot water bath for 20 min to allow for complete dissolution of the particles. After cooling down to room temperature, Fe^{3+} was reduced by hydroxylamine hydrochloride at pH 4 and the concentration of iron was determined by complexometry with *o*-phenanthrolin. The absorbance of the solution was read at 510 nm on a diode/array UV/visible spectrophotometer (Hewlett Packard, 8452 A).

In Vivo Characterization of MSM

Liver perfusion. Wistar male rats weighing about 150 g were anesthetized with 0.2 g of urethane (U-2500, Sigma, St. Louis, MO) and injected with 500 U.I. of heparin (Novo Nordisk, Bagsvaerd, Denmark) into the vena cava. Two perfusion protocols were set up. In the first one, for the recycling mode experiments, the livers were isolated as described in (*8*) and perfused at a constant flow rate of 28 ml/min through the portal vein. A volume of 160 ml of K-H buffer at 37°C was used. The second protocol, for the single pass mode experiments, was the same as protocol 1, except that the perfusate expelled from the liver was not allowed to recirculate. All the animal experiments fulfill the requirements of the Ethical Committee of our institution.

Relaxometric measurements. For the recycling mode experiments (protocol 1), the livers were perfused in a 25 mm o.d. NMR Pyrex tube and placed in the 3 cm diameter-coil of a spin analyzer PC-100 (Bruker, Karlsruhe, Germany) working at 0.235 T and thermostated at 37°C. The liquid flowing out of the liver was continuously pumped at the bottom of the NMR tube to avoid any contamination of the NMR signal arising from the tissue water protons. The measurements were performed through CPMG sequences with three different values of TE (0.2, 0.8, and 4.0 ms). Four livers were examined for each echo time. Three control measurements were performed prior to the addition of the contrast agent to the 160 ml of perfusate. The doses used were 300, 750, or 1500 μg, corresponding to 0.033, 0.084, and 0.167 mM, respectively, of iron. Thereafter, the livers were examined every 2 min during the first 20 min and every 15 min afterwards.

The single pass mode experiments (protocol 2) were set up in order to evaluate the relative contributions of intravascular and intracellular particles to the hepatic proton relaxation. Five livers were perfused for the first 20 min with the contrast agent at the dose of 0.027 mM of iron at a flow rate of 28 ml min^{-1}, and then rinsed with fresh buffer to eliminate any circulating particles from the sinusoids. Throughout this experiment, the hepatic tissue was studied by a CPMG sequence with TE = 0.8 ms.

Pharmacokinetics

In the protocol of recirculating mode, aliquots of 0.3 ml were collected from the reservoir of perfusate at times 5, 10, 15, 30, 45, 75, and 90 min for relaxometric dosage in order to follow the decay of the MSM concentration in the perfusion fluid. The evolution of the concentration was fitted according to the bicompartmental pharmacokinetics model (*10*). Four parameters were obtained from this fitting: the clearance Cl = dose/AUC_0^∞ (ml/min), the volume of distribution Vd (ml), the half-life time $t_{1/2}$ (min), and the extraction coefficient E = Cl/Q. AUC_0^∞ is the area under the curve representing the dose as a function of time, and Q is the perfusion flow rate.

Electron Microscopy

After 15 min of recycling perfusion with MSM at the dose of 0.084 mM of iron, the liver was rinsed and fixed by the single-pass perfusion of glutaraldehyde (2%) in a cacodylate buffer. The samples were postfixed with osmium tetroxide and dehydrated by successive immersions in alcohol baths. The slides were counterstained with uranyl acetate and lead acetate. The micrographs were performed at 40 kV on a Zeiss EM10C/CR microscope.

RESULTS AND DISCUSSION

Figure 1 shows the evolution of the proton transverse magnetization of a K-H solution containing MSM at the dose of

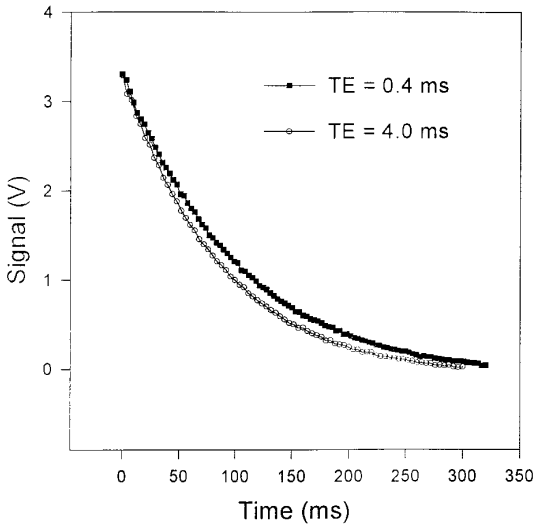

FIG. 1. Evolution of the transverse magnetization of a K-H solution of MSM (0.07 mM of iron) recorded with a CPMG sequence, two echo times (0.4 ms and 4.0 ms), TR = 3s, and T = 37°C.

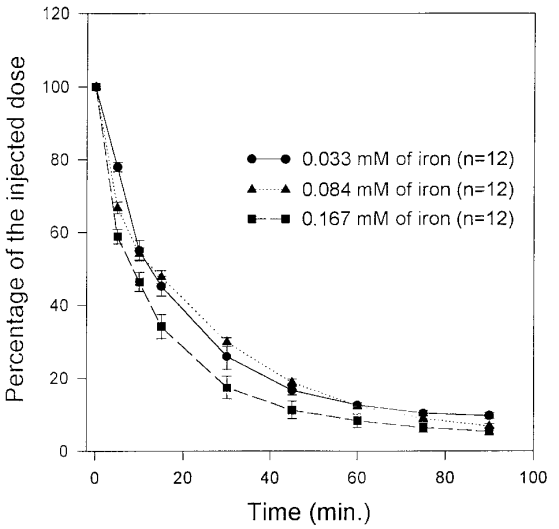

FIG. 3. MSM clearance from the 160 ml recycled volume of the perfusate for three administered doses: 0.033, 0.084, and 0.167 mM of iron.

0.067 mM of iron with TE = 0.4 and 4 ms. For both values of TE, no ultrafast component of the magnetization is observed and the regime of the decay is monoexponential (the slightly faster decay at TE = 4 ms reflects local macroscopic inhomogeneities). These results give evidence of the good dispersion and stability of the colloidal contrast agent in the perfusion liquid.

Figure 2 depicts the linear evolution of R_2 increment of the K-H solution as a function of MSM concentration at 37°C and 20 MHz. From the slope of this straight line, a transverse relaxivity r_2 of 182 $s^{-1}mM^{-1}$(Fe) is estimated which will be used to convert the R_2 values of the perfusion solutions into their iron concentrations.

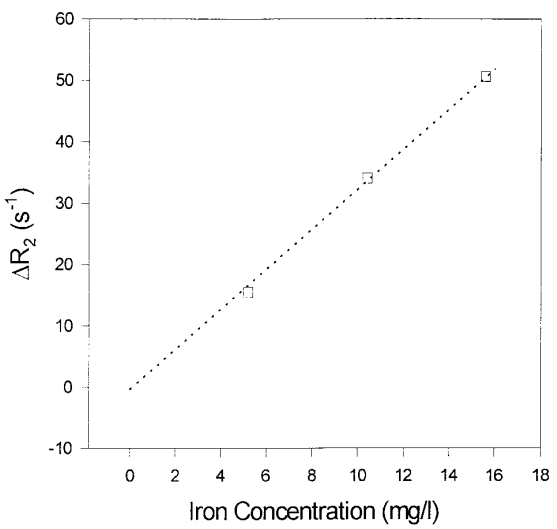

FIG. 2. Increment of the transverse relaxation rate of K-H solutions induced by various doses of MSM (TE = 0.4 ms, 0.47 T, 37°C).

Figure 3 compares the kinetics of the clearance of the particles from the perfusate as a function of the administered dose (recycling mode). For each dose, 12 sets of data were available since the experiments were carried on 4 livers for each of the 3 TE selected to assess the relaxation regime of the organ. The evolutions of the MSM concentration in the perfusate are similar and biexponential, suggesting a two-step mechanism for the clearance of this contrast material. The initial decay corresponds to the distribution phase of the product in the volume of perfusate after its delivery. The second process is slower and accounts for the elimination phase.

For these recirculating experiments, the values of the pharmacokinetics parameters obtained from the fitting of the experimental curves are collected in Table 1. From 0.033 mM up to 0.167 mM of iron, the mechanism of uptake of MSM by Kupffer cells follows the same nonsaturated trend. As for the single-pass perfusions, the clearance is about 7.5 ml/min at the dose of 0.027 mM of iron (*11*).

We previously demonstrated (*12*) that for larger doses (>0.536 mM of iron), the distribution phase of the uptake still follows an exponential regime while the elimination phase progressively tends to a linear behavior, a trend which reflects the saturation of the uptake mechanism likely to arise from the

TABLE 1
Pharmacokinetics Parameters of the Hepatic Uptake of MSM Measured with the Recirculating Perfusion

Dose (mM of iron)	0.033	0.084	0.167
AUC_0^∞ (min μg iron/l)	63	116	195
Cl (ml/min)	4.8	6.5	7.7
Vd (ml)	158.7	163.8	160.8
$t_{1/2}$ (min)	13.0	13.5	8.5
E	0.17	0.23	0.28

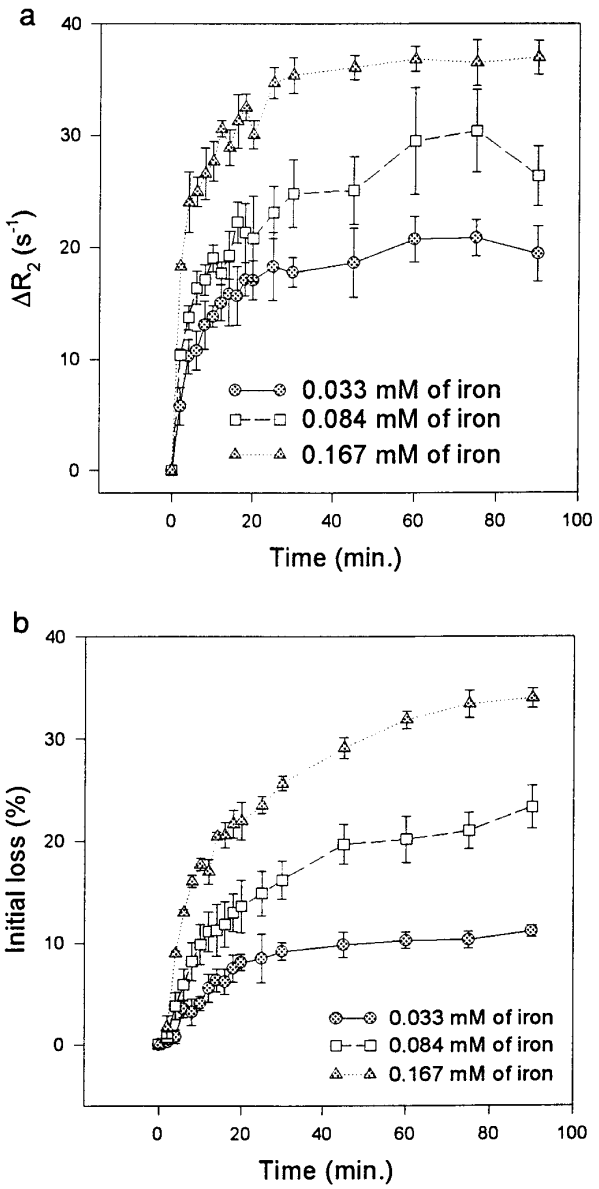

FIG. 4. Influence of the administered dose of MSM on the evolution of R_2 and IL of the perfused liver (TE = 4.0 ms).

overload of membrane receptors. It has to be mentioned that, at any time and concentration, the evolution of the transverse magnetization of the perfusion liquid remains characteristic of a dispersed superparamagnetic material. This confirms the stability of the contrast agent during the perfusion in spite of the fact that the recirculating fluid accumulates by-products, such as proteins released by the liver, which could alter the structure or the stability of the microspheres.

Figures 4a and 4b, respectively, compare the evolutions of R_2 and of the fast magnetization decay (the "initial loss" IL) of the tissue water protons during the perfusion with the contrast agent at various doses and for TE = 4.0 ms. The graphs show a dose-dependent increase of R_2 and IL during the perfusion with MSM.

On the other hand, the evolutions of R_2 (Fig. 5a) and IL (Fig. 5b) become dependent on the TE length quickly after MSM administration. A rapid increase of both parameters is observed within the first minutes and then R_2 and IL reach plateaus, the amplitudes of which depend on both the dose of MSM and on TE length.[2] It is worth noting that even when a steady level is reached, the liver is still taking up circulating microspheres (see Fig. 3). For more clarity, the amounts of ingested iron extrapolated from the pharmacokinetics data are included as the top axis in Fig. 5, assuming that all particles which have disappeared from the perfusate have been retained in the liver. This phenomenon of apparent saturation of R_2 occurs approximately 30 min after administration of the contrast agent, when more than 80% of the dose has already been internalized. The plateau values are maintained until the end of the experiment, when the concentration of MSM in the perfusate is very low as indicated by the relaxometric dosage. It is interesting to note that in the context of these experiments, the relaxation effects induced by a given amount of internalized particles are independent of the administered dose and therefore of the initial concentration of the perfusate. As an example, a quantity of 240 μg of iron which corresponds to 80, 32, and 16% of the initial doses of 300, 750, and 1500 μg is respectively internalized after 38, 5, and 2 min, inducing the same IL and R_2 enhancement.

These evolutions have thus to be related to an alteration of the transverse relaxivity of the contrast agent. If one considers the lowest administered dose, the liver has taken up 300 μg of iron at the end of the experiment (see Fig. 3). For a liver weight of 7 g and a water content of approximately 5 ml, the final iron concentration is then about 1 mM. If the particles were to be well dispersed and freely accessible to water molecules as in aqueous solutions, then the R_2 increment should be about 182 s^{-1} (1 mM$*r_2$) and independent of TE. However, the measured value is about 6 to 20 s^{-1}, depending on TE duration (data not shown). As previously demonstrated by computer simulations (*4, 13, 14*) and experimental data (*6*), this evolution of the water transverse relaxation and its sensitivity to echo time are associated with an increase of the dimensions of the magnetized clusters. As shown in the figures, this happens so quickly after the onset of the uptake process that a disruption of the coating can be excluded. It has indeed been shown that the digestion of the starch matrix and the redistribution of the iron to the erythrocytes require several days (*15*).

Figure 6 shows the electron micrograph of an isolated liver (original magnification, ×12,000), 15 min after administration of the contrast agent at the dose of 0.084 mM of iron. The picture displays a Kupffer cell in the lumen of a sinusoid. Numerous particles fixed on the cytoplasmic membrane are observable. Because of the electron density of the iron oxide cores, the particles appear very dark. Near the nucleus (N), the presence of a phagolysosome (P, 1 to 2 μm) containing internalized magnetic microspheres is noticed. This physical distri-

[2] Although the strong TE dependence of the transverse relaxation of water tissue would favor the use of R_2*, the symbol R_2 will be kept throughout the text

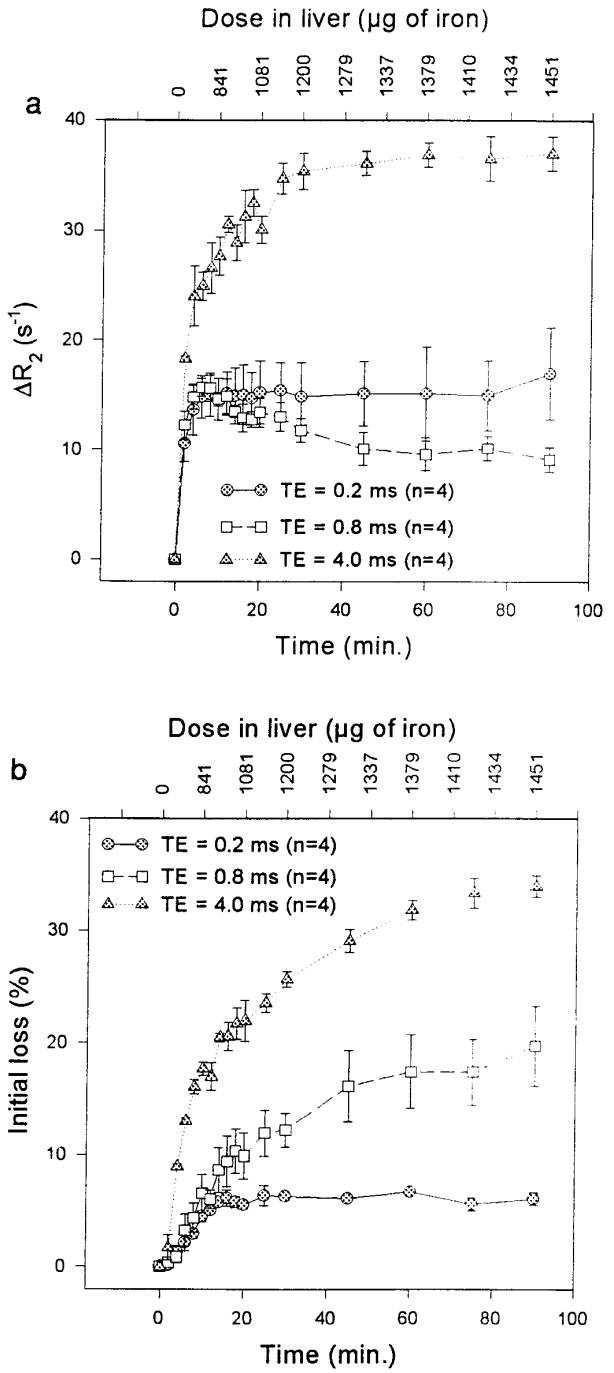

FIG. 5. Influence of the echo time on R_2 (a) and IL (b) of hepatic tissue during the perfusion with MSM (0.167 mM of iron). Top axis expresses the amounts of ingested iron extrapolated from the pharmacokinetics.

bution of the superparamagnetic particles could explain their passage from the dispersed state to an agglomerate system. The vacuoles filled with microspheres behave as large agglomerates. The *in vivo* hepatic uptake and cellular distribution of AMI-25, a product similar to MSM, has been studied by Wisse *et al.* (*16*), who reported from a single low dose experiment (1.13 mg Fe/Kg) that the lysosomal compartment is reached 15 min after administration. In our experiments, this could explain the appearance of the plateaus of R_2 values 20 min after administration of MSM to the perfusion fluid.

These results also demonstrate that the tissue relaxation rate R_2 cannot be directly related to the concentration of superparamagnetic particles in the tissue, as is the case in solution.

Finally, the respective contributions of intravascular and intracellular particles to the transverse relaxation of the hepatic tissue were analyzed from the single pass mode experiments. The contribution arising from the vascular particles is expected to be low since the sinusoidal space only accounts for 10% of the total volume of the liver while the cellular compartment represents 86% of the organ (*17*). The remaining 4% corresponds to the biliary tract and the Disse space. Considering these volumes and assuming a fast water exchange between the vascular and cellular compartments, R_2 of the liver would result from the sum of two contributions: $R_{2\,\text{liver}} \sim 0.10R_{2\,\text{vascular}} + 0.86R_{2\,\text{cellular}}$. Hence, even at the highest contrast agent concentration used in this study (0.167 mM) and assuming that all the particles remain intravascular, the maximum $R_{2\,\text{liver}}$ increment should be 3 s^{-1} (0.1 × 0.167 mM × 182 $s^{-1}mM^{-1}$). Figures 7a and 7b show the evolutions of R_2 and IL observed during the perfusion with MSM and during the subsequent washout of the sinusoids. As already observed in the case of recycling perfusions, R_2 and IL rapidly increase after administration of the contrast agent and reach steady-state values. At the concentration of 0.027 mM (Cl = 7.5 ml/min), close to the dose of 0.033 mM of iron in recycling conditions (160 ml / clearance = 7.7 ml/min), the plateau values observed at TE = 0.8 ms for R_2 and IL are, respectively, about 10 s^{-1} and 10% in closed circuit and 10 s^{-1} and 6% for open circuit. These plateau values are preserved even during the washout of the sinusoids, suggesting that in these conditions of moderate concentration and short TE, the increment of R_2 and IL in the hepatic tissue is mainly provided by the fraction of tightly bound and ingested particles while intravascular contribution to T_2 and/or T_2^* is negligible.

Of course, in our isolated and perfused liver system, the volume of perfusate is 160 ml, much larger than the blood volume of a living rat (±10 ml). Thus, for the same administered dose, the concentration of particles is much lower in our protocols. This might explain the minor effects of the particles from the vascular compartment in our experiments. Mandeville *et al.* (*18*), using vascular iron oxide agents, have recently demonstrated that, *in vivo*, liver relaxation is consistent with rapid water exchange between vascular and extravascular compartments. As long as they remain in circulation, the particles attenuate liver signal predominantly through a vascular mechanism. After the internalization process takes place, the extravascular compartment dominates the relaxation as a result of the accumulation of the agent in the Kupffer cells. Since they used ultrasmall particles (about 17 nm diameter) with a very long vascular remanence ($T_{1/2}$ = 4 h), the authors were able to observe successively the vascular and the extravascular effects on relaxation. In contrast, in our experiments, we have used larger particles (about 200 nm diameter) which are rapidly

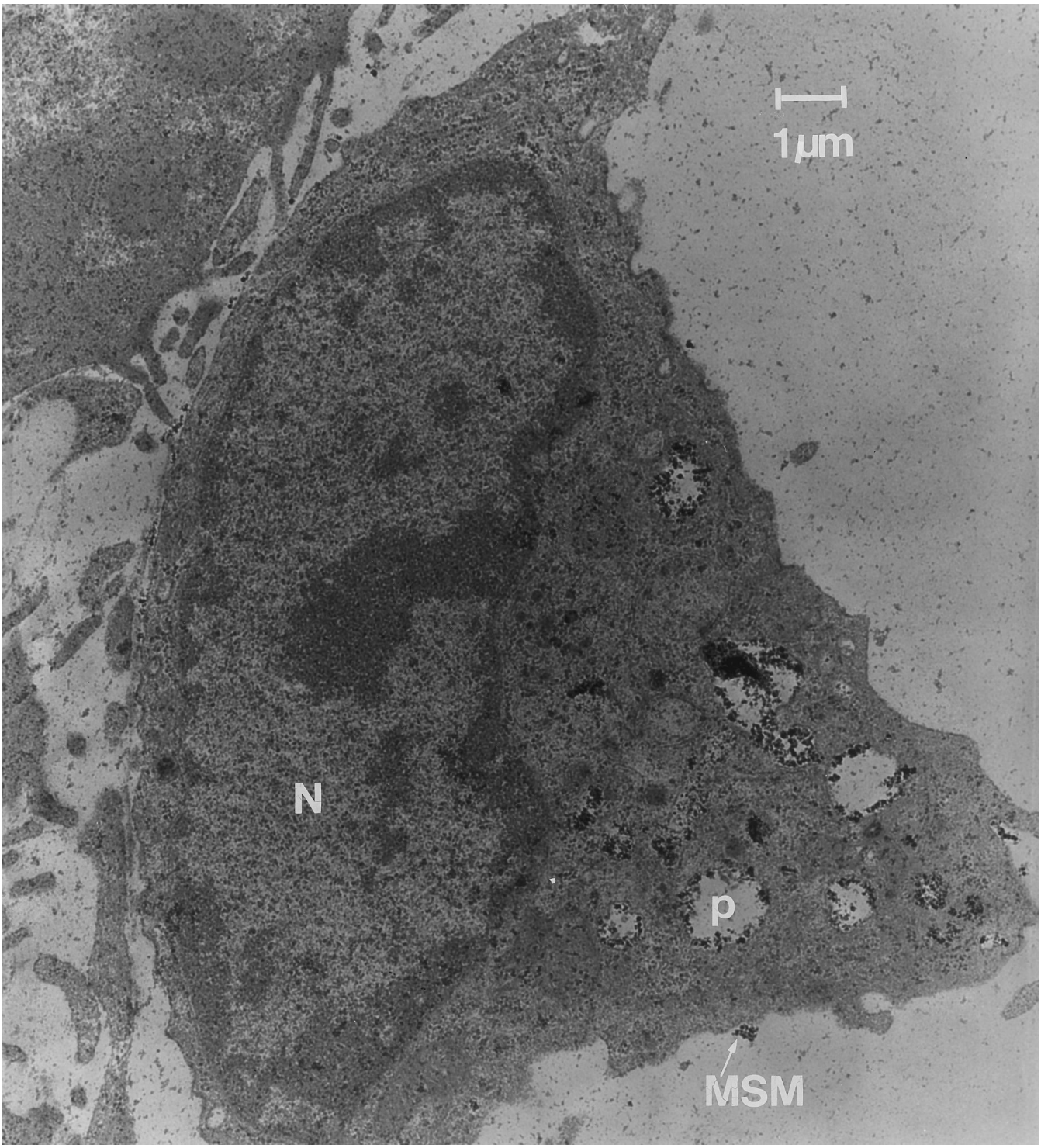

FIG. 6. Electron micrograph of a Kupffer cell from an isolated rat liver perfused with MSM (M = 14000). MSM, microspheres fixed to the cytoplasmic membrane; N, nucleus; P, phagolysosome containing internalized particles.

cleared by Kupffer cells ($T_{1/2} \approx 12$ min). Under such conditions, the ingested particles dominate the relaxation very quickly after their administration, preventing the observation of the vascular effect.

CONCLUSIONS

In the context of the *in vitro* characterization of superparamagnetic particles, it had been previously shown that the

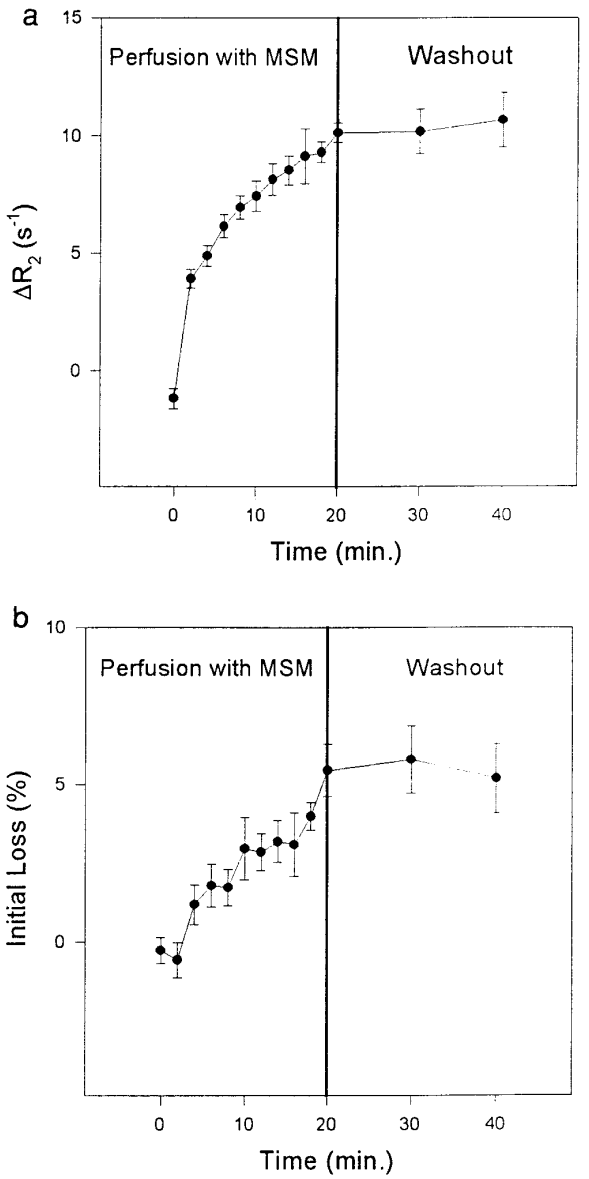

FIG. 7. Evolutions of R_2 (a) and IL (b) of the isolated liver ($n = 5$) during the perfusion with MSM (0.027 mM of iron) and during the washout of the sinusoids (TE = 0.8 ms).

evolution of the transverse component of the magnetization was a valuable parameter to assess the extent of clustering of the magnetic crystals (*6*).

In the present work carried on excised and perfused liver, we have demonstrated that this information was also useful to follow up the aggregation of small particles *in vivo.* The hepatic uptake of magnetic starch microspheres, a well-dispersed superparamagnetic system, induces the physical agglomeration of the particles in intracytoplasmic vacuoles. This phenomenon might have two opposite outcomes, depending on the imaging pulse sequence and the delay of the acquisition. On the one hand, the rapid initial loss of magnetization should be beneficial for the contrast of gradient echo images collected with short TE. On the other hand, the agglomeration process might considerably reduce the efficacy of the material in terms of pure transverse relaxivity r_2 in the context of spin echo imaging sequences (*4*, *19*). Precise quantitation of these effects, however, needs a detailed knowledge of both the cellular distribution and the extent of the agglomeration of the particles.

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