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A Mössbauer spectrometry study of iron in hepatic and splenic tissues.
Preliminary results

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Résumé. — La ferritine, protéine de réserve du fer chez les mammifères, est constituée d'un noyau d'hydroxyde de fer entouré d'une couche protéique. Plusieurs études effectuées par spectrométrie Mössbauer sur les molécules de ferritine extraites de rate de cheval montrent que le noyau de fer présente des propriétés superparamagnétiques et que l'effet Zeeman nucléaire, caractéristique d'un ordre magnétique, apparaît au-dessous de températures 40-30 K. Parmi les organes et les tissus, ce sont le foie et la rate qui présentent le contenu en fer le plus élevé, principalement sous forme de ferritine et d'hémossidéline. Dans le présent travail, des spectres Mössbauer d'échantillons lyophilisés de rate et de foie de cheval, de bœuf et de veau ont été réalisés aux températures du laboratoire, de l'azote et de l'hélium liquide. Conformément aux travaux antérieurs on trouve sur les échantillons lyophilisés de rate de cheval, que les spectres à 4,2 K sont essentiellement caractérisés par un effet Zeeman du noyau de fer. Par contre on observe dans les spectres d'échantillons de foie lyophilisés de cheval, de bœuf et de veau, à une température aussi basse que celle de l'hélium liquide, un doublet Fe^{3+} , représentant une fraction importante du spectre Mössbauer en sus du sextuplet Zeeman.

Abstract. — Ferritin, mammalian iron reserve protein, is made of a ferric hydroxide core enclosed in a protein shell. Several Mössbauer spectrometry studies have been carried out on ferritin molecules extracted from horse spleen. They demonstrate the existence of superparamagnetic properties in the iron core and the coming out of a nuclear Zeeman effect, characteristic of a magnetic order, below 40-30 K temperatures. Among the organs and tissues of the body, the liver and spleen usually rank highest in iron content, mainly as ferritin and hemosiderin. In the present work, Mössbauer spectra of lyophilized samples of liver and spleen from horse, beef and calf have been recorded at room, liquid nitrogen and liquid helium temperature. In accordance with the previous works we find for the lyophilized horse spleen samples, the spectra at 4.2 K are mainly characterized by a Zeeman effect of the iron nucleus. On the other hand, in lyophilized samples of horse liver, even at liquid helium temperature, a doublet corresponding to Fe^{3+} is observed, in addition to the Zeeman sextuplet, representing an important fraction of the Mössbauer spectrum.

1. **Introduction.** — It is in the bone marrow and red blood cells that the major concentration of iron is found in the adult human, 60-75 % of the total, principally in the form of circulating hemoglobin. But among the organs and tissues of the body, the liver and spleen usually rank highest in iron content, containing 7-15 % of the total iron in the human body [1]. Table I is taken from Zuyderhoudt *et al.* [2] to show the distribution of iron in normal human liver according to its biochemical composition. The major part is found in the form of ferritin and a lesser amount as hemosiderin. These two constitute about 90 % of the iron in hepatic tissue, the remainder being distributed among various heme enzymes. They form the storage iron of mammalian species and play an essential role in their physiology.

Ferritin is a protein with a molecular weight of about 700.000 which contains up to 30 % of its weight as iron, in the form of an inorganic ferric hydroxide-phosphate polymer consisting of an average of 2 500 atoms of iron enclosed in a protein shell [3].

Table I. — Analysis of iron compounds in biopsy specimens of normal human liver in $\mu g/g$ dry weight.

Total liver iron	Ferritin iron	Haem iron	Non-ferritin depot iron (hemosiderin)
730	457	87	186

When saturated the core contains about 4 500 atoms of iron and has an outer diameter of 80 Å, while the outer diameter of the protein shell is of the order of 120 Å. The term *hemosiderin* is applied to granules containing a variable quantity of iron (representing 29 ± 8 % of their dry weight) and which, contrary to ferritin, is insoluble.

Table II is from a study in the rat by Drysdale and Ramsay [4] showing, in this animal at least, the quantity of ferritin is much greater than that of hemosiderin in the liver, the reverse being true for the spleen. It also

Table II. — *Quantity of iron present in the form of ferritin and hemosiderin in the liver and spleen of normal adult male rats, in $\mu\text{g/g}$ dry weight.*

Organ	Number of animals	Ferritin	Hemosiderin
Liver	46	336 ± 40	96 ± 24
Spleen	46	548 ± 140	$1\,456 \pm 400$

shows that in the rat the total quantity of storage iron is about 5 times greater in the spleen than in the liver.

Mössbauer spectrometry has been used to study both of these components by Blaise *et al.* [5], Boas and Window [6], Fischbach *et al.* [7], Webb and Gray [8], Williams *et al.* [9] and Bauminger *et al.* [10]. The principal finding of these studies was a drastic change in the spectra at temperatures around 35–40 K. Above this temperature the spectra consist of a Fe^{3+} doublet. Below this, a magnetic hyperfine splitting appears, and, at a sufficiently low temperature, the spectrum consists of the six characteristic lines of the nuclear Zeeman effect in iron. This result has been interpreted by supposing that the small particles of the iron of ferritin and of hemosiderin are superparamagnetic and display antiferromagnetic order of the atoms of iron at sufficiently low temperatures. The spectra, at the temperature of liquid helium, can be analyzed in terms of the continuous distribution of the hyperfine magnetic field, which then permits determination of the distribution of the dimensions of the iron particles in a sample [9].

These investigations with Mössbauer spectrometry have been carried out on ferritin or hemosiderin, extracted by various chemical procedures, sometimes after enrichment with ^{57}Fe , and principally on ferritin of horse spleen. Most of research on ferritin has been done with molecules extracted from horse spleen, an organ which in this animal is particularly rich in storage iron. It seemed to us that it would be interesting to avoid the chemical extraction step for ferritin and hemosiderin and to employ the Mössbauer technique for direct measurements on the hepatic and splenic tissues themselves, after a simple conventional lyophilization.

2. Material and methods. — Different samples of liver and spleen from horse, beef and calf were studied. After washing in cold running water for 24 hours, the samples of the organs were immersed in physiological saline in a proportion of 1/3 tissue and 2/3 saline. The mixture was ground in a blender. The mixture thus obtained was sufficiently thick to be placed in a thin layer in a balloon and immersed in liquid nitrogen. It was then lyophilized at -90°C under vacuum of 0.05 torr. Some spectra were obtained on samples of Bovine Liver purchased by the National Bureau of Standards as biological matrix standard reference materials for trace element determination [11].

The content of iron in the different samples of lyophilized tissue, measured by activation analysis, was between 250 and $17\,500\ \mu\text{g/g}$ in the spleen and between 150 and $800\ \mu\text{g/g}$ in the liver. For each of the three types of animal it was higher in the splenic than in the hepatic tissues (Table II). In both the liver and spleen the highest levels of iron were found in the horse.

— The spectra were recorded by means of a constant acceleration device, using a source of 50 mCi of ^{57}Co in rhodium and a Harwell proportional counter. The absorber of 8 mm thickness and 18 mm diameter consisted of 1.2 g of lyophilized tissue. The content of iron per square centimeter was from 80 to $400\ \mu\text{g/cm}^2$ for the samples of liver and from 100 to $8\,000\ \mu\text{g/cm}^2$ for the spleen. The spectra were obtained with the absorber at three temperatures : room temperature, liquid nitrogen, and liquid helium.

— The counting times were between 3 days and 2 weeks depending on the content of iron in the absorber. The total number of counts per channel in the 400 channel analyzer was between 10^7 and 6.5×10^7 .

3. Results. — Figure 1 shows the Mössbauer spectrum at room temperature and at 4.2 K of a typical sample lyophilized horse spleen having an elevated iron content of $17\,200\ \mu\text{g/g}$. At room temperature the spectrum consists of an Fe^{3+} doublet with isomeric shift $\delta = 0.32 \pm 0.05\ \text{mm/s}$ relative to iron metal and a quadrupole splitting $\Delta = 0.72 \pm 0.05\ \text{mm/s}$. At liquid helium temperature the spectrum consists of a sextuplet of broadened and asymmetrical lines, characteristic of a magnetically ordered material, a distribution of magnetic field, and an isomeric shift :

Fig. 1. — Mössbauer spectrum of a lyophilized horse spleen sample. Top) At room temperature. Bottom) At 4.2 K.

$\delta = 0.51 \pm 0.05$ mm/s. This result is completely in agreement with the literature on Mössbauer parameters of ferritin and hemosiderin [5-10]. The characteristic increase of the isomer shift at low temperatures is observed. Between the two inner lines of the spectrum at 4.2 K an additional component can be seen, which we ascribe to the presence of traces of iron in the beryllium windows of the cryostat.

The results obtained are significantly different from those obtained with lyophilized hepatic tissue. In effect, at liquid helium temperature, the spectrum obtained shows, in addition to the sextuplet, an important doublet. Figure 2 shows typical results for a sample of horse liver, for which the content of iron of 837 $\mu\text{g/g}$ is the highest we have encountered in this organ. The upper part of figure 2 corresponds to the directly recorded spectrum at 4.2 K. Taking into account the low iron content of the sample, this spectrum results from the superposition of the spectrum of the sample itself and that due to traces of iron contained in the beryllium windows of the cryostat. The spectrum of the latter is shown in the middle part of the figure. Finally, the spectrum in the lower part, obtained from the difference of the two preceding ones, corresponds to the spectrum of the sample itself. The latter shows the sextuplet corresponding

to ferritin observed in the spleen, plus a doublet Fe^{3+} with an isomeric shift $\delta = 0.50 \pm 0.05$ mm/s and a quadrupole splitting $\Delta = 0.63 \pm 0.05$ mm/s. Within the limits of precision of the measurement, the area under this doublet corresponds to 65 % of the total area under the spectrum. Figure 3 shows the spectrum of the same sample obtained at room temperature. It corresponds to an Fe^{3+} doublet with isomeric shift $\delta = 0.35 \pm 0.05$ mm/s and a quadrupole splitting $\Delta = 0.63 \pm 0.05$ mm/s.

HORSE LIVER at Room Temperature
iron:837 ppm

Fig. 3. — Mössbauer spectrum of lyophilized horse liver sample at room temperature.

Fig. 2. — Mössbauer spectrum of a lyophilized horse liver sample at 4.2 K. Top) Sample spectrum directly recorded. Middle) Spectrum of the cryostat windows iron traces. Bottom) Difference between the two previous spectra corresponding to the only lyophilized horse liver sample.

4. Discussion. — Niitsu and Listowsky, utilizing density gradient centrifugation [12, 13], estimated that the dominant component of ferritin extracted from horse spleen and rat liver contained approximately 2 000 atoms of iron per molecule of protein. For such a number of atoms the theory of superparamagnetism shows that the Mössbauer spectrum at 4.2 K should consist essentially of a sextuplet. This is indeed found in the lyophilized samples of horse spleen. Since we have not been able to study lyophilized samples of rat liver, it is not possible to state whether the corresponding Mössbauer spectra would show a Fe^{3+} doublet in addition to the ferritin sextuplet. However we are able to demonstrate the appearance of such a doublet in the Mössbauer spectra at 4.2 K in lyophilized sample of horse, beef and calf liver. How can we explain this result ?

Three hypothesis can be advanced. — 1) Lyophilization profoundly perturbs the ferritin molecules, to such an extent as to destroy the magnetic ordering, even at low temperatures. This hypothesis is hardly consistent with the usual view that lyophilization does not seriously disturb the molecules.

2) The dimensions of the iron core in some fraction of the ferritin molecules in horse, beef and calf hepatic tissues are so small that no magnetic order appears at 4.2 K. A rough calculation, based on the

theory of superparamagnetism, shows that the number of atoms of iron per molecule of ferritin should then be of the order of 300. It remains to be explained why, in the three types of animal studied, this number should be much smaller than in the case of the ferritin in rat liver.

3) Bauminger *et al.* [14] in the bacterium *Escherichia Coli*, Dickson and Rottem [15] in *Proteus Mirabilis*, have discovered a protein of storage iron for

which they have not been able to demonstrate a magnetic transition down to temperature below 4.2 K. The existence of an iron reserve protein of this type, different from ferritin, in hepatic tissues of horse, beef and calf would explain our results. This would be completely unexpected. Only a very precise study of the variation of Mössbauer parameters as a function of temperature might serve to confirm or disprove such an hypothesis.

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