An Analysis of Iron-Dextran Complexes by Mössbauer Spectroscopy and Positron Annihilation Technique

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ABSTRACT

The combination of two modern spectroscopic techniques was used for comparative microstructural analysis of several iron-dextran complexes which were used or elaborated as drugs for treatment of iron-deficiency anemias. Slight variations of the iron electronic and magnetic structure in different iron-dextran complexes were detected by Mössbauer spectroscopy. The differences of iron core structure and size were shown in studied complexes also. Variations of dextran structure were detected by the positron annihilation technique for investigated iron-dextran complexes. These results were considered with histological and histochemical data for iron-dextran complexes to compare the differences of their microstructural variations and biochemical pathways.

INTRODUCTION

Iron is one of the most vitally important elements. Iron ions are in the active sites of various enzymes, electron and oxygen transport proteins. Therefore, iron deficiency due to several pathological states of the body leads to iron-deficiency anemias. For treatment of such anemias special iron-containing drugs are needed. These drugs should contain iron complexes in soluble form at physiological conditions which could be easily accumulated in the body.

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Iron-dextran complexes are pharmaceutically important synthetic models of iron storage protein ferritin. It is well known that ferritin consists of a hydrous ferric oxide ($Fe_2O_3 \times nH_2O$) core with a multisubunit protein shell [1]. In contrast, iron-dextran complexes consist of a dextran (linear (1-6)-poly-D-glucose) shell surrounding a hydrous ferric oxide core. Some iron-dextran complexes are used (Imferon) or elaborated as drugs for treatment of iron-deficiency anemias. However, these complexes have different effects in the body. It is possible that these differences could be related to structural variations of iron-dextran complexes. Therefore, we used modern physical techniques, Mössbauer spectroscopy, and positron annihilation for comparative analysis of some iron-dextran complexes. Earlier Mössbauer and EXAFS studies had been made for iron-dextran complexes (Imferon) in comparison with iron-storage proteins to analyze iron core structures [2, 3]. In the present work we continue our previous studies (see [4, 5]) of both iron core and dextran shell structures as well as biochemical pathways of iron-dextran complexes.

EXPERIMENTAL

Materials

Several samples of iron-dextran complexes were studied. Samples of Imferon (Benger, U.S.) were chosen as widely adopted drugs. Samples of Dextrafer (USSR) were taken as examples of ineffective drugs. Four elaborated samples of iron-dextran complexes with different molecular weights of dextran and numbered as IDC1, IDC2, IDC3, and IDC4 were studied also. Samples of IDC1 and IDC2 were prepared using 6–10% solutions of dextran with molecular weights of 22,000 and 16000–18000 Da, respectively, by adding $FeCl_3$ solution (dextran: $Fe^{3+} = 1:0.2$ g/g) and NaOH to produce a mixture with pH ~ 2.2–2.3. This mixture was heated at 60°C for 20 min then pH was changed to ~11.5 and the mixture was heated again in the boiling water for 20 min. The precipitated complex was dissolved at pH ~ 3.0 and filtrated using Millipore and Amicon YM5 filters (0.8, 0.6, 0.45 μm). After adding NaCl to 0.9% concentration the solution was filtrated with a 0.22 μm filter and sterilized. Samples of IDC3 were prepared by adding NaOH solution to the mixture of dextran with molecular weight of 8000 Da and $FeCl_3$ (dextran: $Fe^{3+} = 1:0.2$ g/g), pH ~ 12. The precipitated complex was dissolved by heating at 75°C for 20 min and filtrated by the same procedure as mentioned above. Samples of IDC4 were prepared in distilled water the same way as IDC3 using dextran with molecular weight of 5800 Da.

All samples of iron-dextran complexes were in physiological solution (0.9% NaCl) except IDC4 and contained 50 mg Fe/ml (Imferon and Dextrafer), 52 mg Fe/ml (IDC3), 13.8 mg Fe/ml (IDC2, IDC4), and 11.7 mg Fe/ml (IDC1). In addition, samples of Imferon, Dextrafer, IDC1, IDC3, and IDC4 were prepared in lyophilized form.

Mössbauer Spectroscopy

Mössbauer spectra were measured with the standard constant acceleration spectrometer using 511 channels of multichannel analyzer. Stability and accuracy of this spectrometer during long time measurements have been shown in

our previous studies of hemoglobin [6, 7]. The 2×10^9 Bq ^{57}Co(Cr) source of Mössbauer radiation was used at room temperature. Standard absorbers of α-Fe and sodium nitroprusside were used at large and small velocity ranges, respectively. Isomer shifts are given relative to α-Fe at room temperature. Samples of iron-dextran complexes in frozen solutions were measured at 87 K while samples of lyophilized iron-dextran complexes were measured at 87 and 295 K. All samples had natural abundance of ^{57}Fe nuclei ($\sim$2.19%). Effective thickness of samples varied from 3 to 33 mg Fe/cm^2. This wide range of effective thicknesses depended on the sample thickness and iron concentration in iron-dextran complexes. Mössbauer spectra were computer-fitted by the least-squares method and Mössbauer parameters (line-width at a half maximum Γ, isomer shift δ, quadrupole splitting ΔE_Q, and hyperfine magnetic field H_{eff}) were evaluated.

Positron Annihilation

Positron annihilation study was made using a conventional long-slit spectrometer of angular correlation described earlier [8]. The 4×10^8 Bq ^{22}Na was used as a positron source. Angular resolution was $\sim$0.8 mrad. Samples of iron-dextran complexes in 0.9% NaCl solution with iron concentrations of 50 and 10 mg/ml were measured at room temperature. To obtain the concentration of 10 mg Fe/ml initial solutions of iron-dextran complexes were diluted with respective volumes of physiological solution. Angular correlation spectra were computer-fitted by a program PAACFIT [9] and positron annihilation parameters (line-width at a half maximum of the wide Γ_W and narrow Γ_N spectral components and relative intensity of the narrow component I_N) were evaluated.

Histochemical and Histological Analysis

Shenshilla rabbits weighing 2.0–2.5 kg and white mice weighing 18–20 g were used for intravenous injections of sterile nontoxic iron-dextran complexes. The samples of Imferon and Dextrafer (0.4% Fe solutions) were injected into rabbits three times (10 ml/kg body weight) while samples of IDC1, IDC2, IDC3, and IDC4 were injected once into mice (0.5 ml of 1% Fe solutions per mouse). Animals were killed in a period from 3 hr to 70 days. Samples of liver, lung, heart, spleen, kidney, and lymph nodes were fixed in neutral phormaline and processed in ordinary ways. The well known Perls method was used for iron determination. The Perls reaction produces a blue color with different intensity of slide areas containing different quantities of iron. Painted slides were analyzed with a light microscope.

EXPERIMENTAL RESULTS

Mössbauer Spectra

Mössbauer spectra of iron-dextran complexes in frozen solutions at 87 K are shown in Figure 1. Their parameters as well as those of lyophilized samples are given in Table 1. It was found that Mössbauer spectra and parameters of corresponding iron-dextran complexes in frozen solution and in lyophilized form are identical. Based on these results we could conclude that the lyophilization process did not effect the iron core structure in iron-dextran complexes. There-

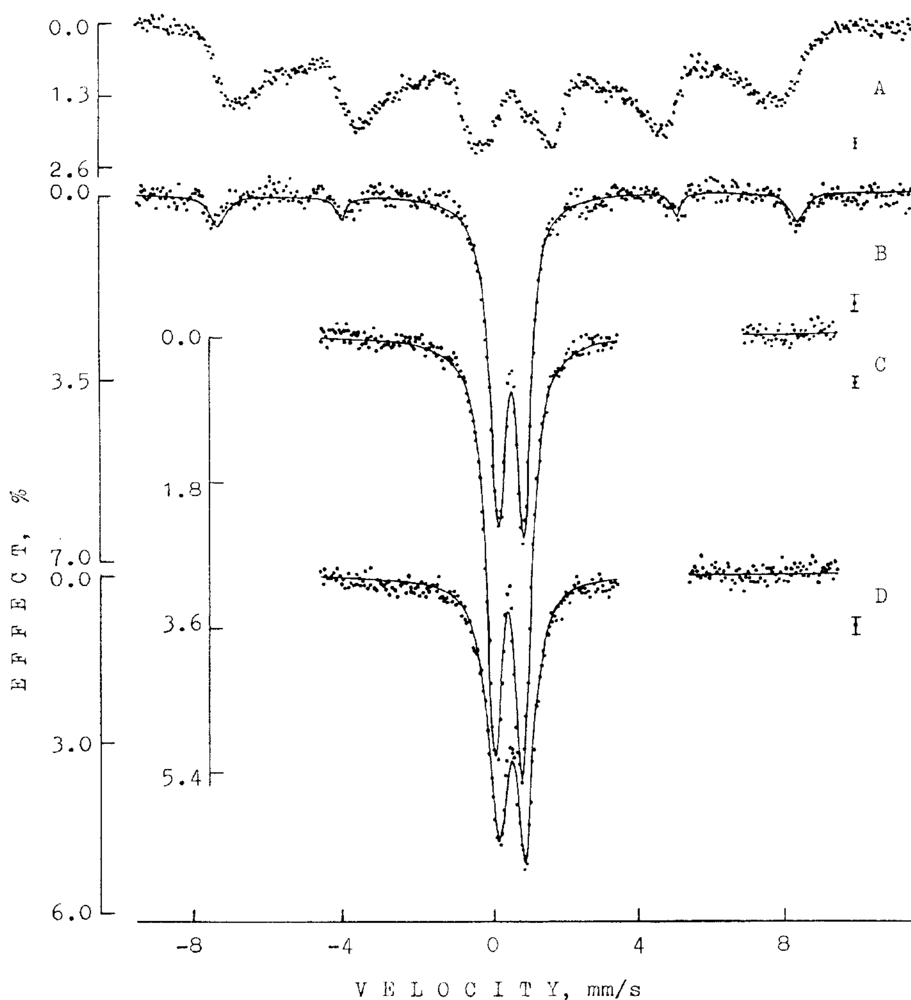

FIGURE 1. Mössbauer spectra of frozen solutions of iron-dextran complexes: (A) Dextrafer; (B) Imferon; (C) IDC1, and (D) IDC2 (T = 87K).

fore, we could compare Mössbauer results obtained for lyophilized iron-dextran complexes and frozen solutions at different temperatures.

Mössbauer spectra of Imferon at 87 K consist of quadrupole and magnetically split components. Relative areas of both components are 71% and 29% for frozen solution and 78% and 22% for lyophilized form, respectively. According to previous studies of oxyhydroxides (FeOOH) [10, 11] our results indicate that the iron in Imferon could be in the form of β-FeOOH clusters in the superparamagnetic state at 87 K. An existence of the superparamagnetic state in this case related to a small iron core size because the temperature of the magnetic phase transition depends on the particle size. The smallest particles are in the paramagnetic state at lowest temperatures. Therefore, it is possible to determine the iron core size in Imferon using this phenomenon. Our supposition about β-FeOOH iron core is in agreement with earlier Mössbauer studies

TABLE 1. Mössbauer Parameters of Iron-Dextran Complexes at 87 K

Complex	Form[a]	δ^b, mm/s	ΔE_Q, mm/s	H_{eff}, kOe
Dextrafer	FS		*not fitted*	
Imferon	FS	0.42 ± 0.05	0.72 ± 0.05	—
		0.50 ± 0.05	-0.15 ± 0.05	485.7 ± 1.6
IDC1	FS	0.42 ± 0.05	0.74 ± 0.05	—
IDC2	FS	0.48 ± 0.05	0.74 ± 0.05	—
IDC3	FS	0.48 ± 0.02	0.77 ± 0.02	—
Dextrafer	L		*not fitted*	
Imferon	L	0.41 ± 0.05	0.68 ± 0.05	—
		0.50 ± 0.05	-0.14 ± 0.05	484.0 ± 1.6
IDC3	L	0.45 ± 0.02	0.77 ± 0.02	—
IDC4	L	0.40 ± 0.05	0.76 ± 0.05	—

[a] FS, frozen solution; L, lyophilized form;
[b] isomer shifts are given with respect to α-Fe at 295 K.

[12] and electron diffraction data [13]. It should be noted that previous Mössbauer spectra of Imferon [2, 3] consist of quadrupole split doublet only at this temperature. However, this distinction could be a result of different sources of Imferon production (Merrill-National Laboratories, Cincinnati, U.S. in Ref. [2] and Fisons Ltd., Leicester, U.K. in Ref. [3]). Moreover, low temperature Mössbauer spectra of Imferon samples in Refs. [2] and [3] were different also. It is possible that aging of Imferon could be another reason of magnetically split component in our Mössbauer spectra because an area of this component was found about 13% in the Mössbauer spectrum of Imferon solution measured two months ago.

Mössbauer spectra of Dextrafer at 87 K consist of approximately three magnetically split component and one low intensity quadrupole split doublet. We could not, unfortunately, fit these spectra. However, we note that the values of H_{eff} for magnetically split components in Dextrafer Mössbauer spectra are lower than H_{eff} in Imferon. We could assume that the iron in Dextrafer is at least in three forms of FeOOH clusters with different structure and size in the superparamagnetic and magnetic states at 87 K. It is possible that the iron core in Dextrafer consists of particles like α-FeOOH because such particles are in the magnetic state at high temperatures and the room temperature value of H_{eff} was about 382 kOe [11].

Mössbauer spectra of IDC1, IDC2, and IDC3 consist of quadrupole split component only while the spectrum of IDC4 contains a weak additional subspectrum. The values of quadrupole splittings and isomer shifts are the same within experimental errors for these complexes (see Table 1). We could suppose that the iron in all these complexes is in the paramagnetic state at 87 K either in the form of β-FeOOH clusters with lower size than those in Imferon or in the clusters of γ-FeOOH. The γ-FeOOH particles are characterized by the low temperature of the magnetic phase transition beginning below 77 K and lower values of the magnetic field ($\sim$ 458 kOe at 4.2 K) [10].

Mössbauer spectra of lyophilized iron-dextran complexes at room temperature at the similar quadrupole split doublets (Fig. 2) with parameters given in Table 2. The values of ΔE_Q are close to well known data of β-FeOOH ($\sim$ 0.7 mm/s) while ΔE_Q values for γ-FeOOH were found $\sim$ 0.5 mm/s and

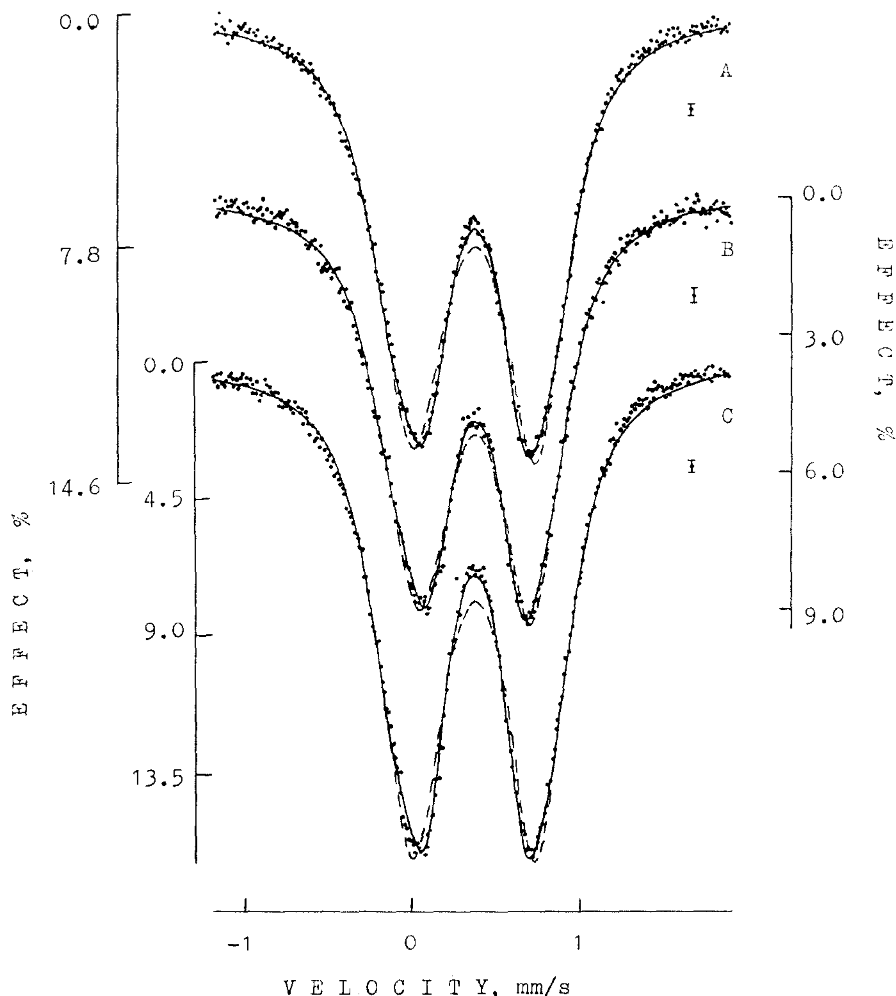

FIGURE 2. Mössbauer spectra of lyophilized iron-dextran complexes at room temperature: (A) Dextrafer; (B) Imferon; (C) IDC4. The dashed and solid lines are the result of the least-square fitting using one and two quadrupole doublets, respectively.

higher values of quadrupole splitting were observed for γ-FeOOH with small particle size (see [10, 11]). The value of quadrupole splitting for Imferon coincides with the value of $\Delta E_Q = 0.68$ mm/s for Imferon (Rallis India Ltd.) [14] and is slightly lower than that of other iron-dextran complexes. This fact could be interpreted as a result of more symmetrical iron core structure in Imferon. The values of ΔE_Q for Dextrafer and IDC appeared to be between ΔE_Q values for Imferon and for Jectofer (Menetes Pharmaceuticals, Panjim), iron sorbitol citric acid dextran complex with $\Delta E_Q = 0.80$ mm/s [14]. However, it should be noted that the room temperature Mössbauer spectra of iron-dextran complexes were fitted unsatisfactorily using one quadrupole doublet as those of other β-FeOOH and γ-FeOOH or ferrihydrites [10, 11]. These spectra were fitted better using two or three quadrupole doublets (see, for example, [15]). We

TABLE 2. Mössbauer Parameters of Iron-Dextran Complexes in the Paramagnetic State (Approximation with One, Two, and Three Quadrupole Doublets)

Complex (Form[a])	T, K	Number of doublets	δ^b, mm/s	ΔE_Q, mm/s	S[c], %	χ^2
Dextrafer (L)	295	1	0.36 ± 0.01	0.73 ± 0.01	100	1437
		2	0.35 ± 0.01	0.94 ± 0.01	46	653
			0.37 ± 0.01	0.59 ± 0.01	54	
		3	0.33 ± 0.01	1.22 ± 0.01	13	528
			0.29 ± 0.01	0.68 ± 0.01	46	
			0.44 ± 0.01	0.68 ± 0.01	41	
Imferon (L)	295	1	0.35 ± 0.01	0.67 ± 0.01	100	1044
		2	0.33 ± 0.01	0.94 ± 0.01	31	641
			0.35 ± 0.01	0.57 ± 0.01	69	
		3	0.33 ± 0.01	1.04 ± 0.01	24	645
			0.35 ± 0.01	0.75 ± 0.01	25	
			0.35 ± 0.01	0.49 ± 0.01	51	
IDC3 (L)	295	1	0.35 ± 0.02	0.75 ± 0.02	100	1320
		2	0.34 ± 0.02	1.05 ± 0.02	33	585
			0.35 ± 0.02	0.63 ± 0.02	67	
	87	2	0.44 ± 0.02	1.07 ± 0.02	40	521
			0.46 ± 0.02	0.63 ± 0.02	60	(1290[d])
IDC3 (FS)	87	2	0.46 ± 0.02	1.11 ± 0.02	30	598
			0.48 ± 0.02	0.66 ± 0.02	70	(742[d])
IDC4 (L)	295	1	0.36 ± 0.01	0.73 ± 0.01	100	2370
		2	0.36 ± 0.01	0.93 ± 0.01	52	946
			0.36 ± 0.01	0.59 ± 0.01	48	

[a] L, lyophilized; FS, frozen solutions;
[b] isomer shifts are given with respect to α-Fe at 295 K;
[c] relative area;
[d] values of χ^2 for one doublet approximation, results given in Table 1.

fitted our room temperature Mössbauer spectra using two quadrupole split doublets as well as spectra of IDC3 at 87 K in both lyophilized form and frozen solution (see Table 2 and solid lines in Fig. 2). Low temperature spectra were fitted better by two quadrupole split doublets also. However, approximation using three quadrupole doublets was not as good for Dextrafer and Imferon (see Table 2) although data for Imferon appeared to be close to those of ferrihydrite core in polysaccharide iron complex [15]. The presence of two or more quadrupole split doublets in Mössbauer spectra of small β-FeOOH particles could be a result of some structural differences including the presence of other modifications of FeOOH or different quadrupole splitting for surface and interior ^{57}Fe nuclei in small particles (see [10, 11, 15]).

Positron Annihilation Spectra

Positron annihilation angular correlation spectra of iron-dextran complexes as well as distilled water (H_2O) and 0.9% NaCl solution were measured and fitted in terms of the wide and narrow spectral components. Parameters of positron annihilation spectra are given in Table 3. The narrow component was attributed

TABLE 3. Positron Annihilation Parameters of Iron-Dextran Complexes in Physiological Solution

Sample	C_{Fe}, mg/ml	I_N, %	Γ_N, mrad	Γ_W, mrad
0.9% NaCl	—	2.74 ± 0.37	2.07 ± 0.23	9.58 ± 0.05
IDC3	50	2.26 ± 0.46	2.11 ± 0.34	9.36 ± 0.06
IDC3	10	3.09 ± 0.52	2.45 ± 0.30	9.40 ± 0.06
Dextrafer	50	3.33 ± 0.49	2.73 ± 0.27	9.74 ± 0.05
Dextrafer	10	4.07 ± 0.57	2.76 ± 0.26	9.46 ± 0.05
Imferon	50	5.18 ± 0.55	2.96 ± 0.20	10.08 ± 0.06
Imferon	10	4.49 ± 0.67	3.01 ± 0.29	9.58 ± 0.06
IDC1	10	4.14 ± 0.57	3.01 ± 0.26	9.81 ± 0.05
H_2O	—	8.62 ± 0.99	3.19 ± 0.23	10.11 ± 0.09

to para-positronium atoms (p-Ps) annihilation in the free volume holes of the samples. The wide component was considered as unresolvable sum of contributions from quasi-free positrons annihilation and pick-off annihilation of ortho-positronium (o-Ps) in the free volume [16].

It was found that the values of Γ_W for Dextrafer and Imferon decreased with Fe concentration decrease while the values of Γ_N were not changed. In contrast, the values of Γ_W and Γ_N for IDC3 were not changed with Fe concentration decrease. Therefore, we could conclude that the values of Γ_N characterized the para-positronium annihilation mainly in the dextran shell. Moreover, the values of Γ_N and Γ_W increased for the sample set of IDC3 ⇒ Dextrafer ⇒ Imferon ⇒ IDC1 (see Table 3) at the iron concentration of 10 mg/ml. It is well known that Γ_N values are related with free volume radius R by the following formula:

$$R \times \Gamma_N = 16.6\ (\text{Å} \times \text{mrad}),$$

which was obtained in terms of the free-volume holes approximation by an infinite depth spherical well [17]. The estimated free volume radii R are given in Table 4. In spite of overlapping estimated errors we could suppose that R decreases with the same sample set as mentioned above. It is possible that dextran branching and crossing density of dextran shell increase with increasing of the molecular weight of dextran. This supposition is in agreement with the results for the free volume radii. Dextran shell friability related with dextran branching and crossing density determines different Cl^- permeability into the

TABLE 4. Free-Volume Radii of Iron-Dextran Complexes Evaluated from Positron Annihilation Spectra

Sample	C_{Fe}, mg/ml	R, Å
IDC3	50	7.87 ± 1.27
IDC3	10	6.78 ± 0.83
Dextrafer	50	6.08 ± 0.60
Dextrafer	10	6.01 ± 0.57
Imferon	50	5.61 ± 0.38
Imferon	10	5.51 ± 0.53
IDC1	10	5.51 ± 0.48

shell (Cl^- ions are partial inhibitors of Ps formation that leads to decrease of the narrow component parameters [18]). Therefore, similar narrow component parameters for IDC3 and 0.9% NaCl solution could be interpreted as higher Cl^- permeability into this iron-dextran complex. Thus, dextran packing in IDC1 is more compact while that in IDC3 is more friable.

Histochemical and Histological Data

Preliminary results showed that iron-dextran complexes were accumulated in rabbits and mice in the same way. Therefore, we could compare the results obtained from these animals. The Perls reaction indicated that studied iron-dextran complexes had different distribution in animal tissues.

Imferon was accumulated within hepatocytes mainly as uniform numerous small blue granules. Endoteleocytes (Kupffer cells) contained small amounts of such granules. The same blue granules were detected in macrophages of other tissues only. In contrast, Dextrafer was accumulated within liver endoteleocytes only as large amorphous masses with various sizes. A congestion of these cells was observed. Accumulation of iron in other tissues was the same as that of Imferon.

The iron from IDC1 and IDC2 samples was accumulated in tissues such as Dextrafer while the iron from IDC3 and IDC4 samples was accumulated in part within hepatocytes and endoteleocytes of liver tissues as well as within macrophages of other tissues.

DISCUSSION

Histological analysis showed that the iron distribution in cells and tissues after Imferon injection was in agreement with other studies [19–21]. This iron was easily accumulated in animals and then stored in the ferritin-like form. On the other hand, the particles of Dextrafer, IDC1, and IDC2 were not accumulated in hepatocytes. Therefore, the iron was removed from the body without storage in ferritin or hemosiderin. However, samples of IDC3 and IDC4 showed intermediate features and some part of the iron was stored in the ferritin-like form. Therefore, we state a question: Would these differences relate with microstructural variations of iron-dextran complexes? For this reason we will consider measured physical parameters.

Positron Annihilation Parameters

The values of Γ_N and Γ_W for Imferon ($C_{Fe} = 50$ mg/ml) appeared to be close to those of H_2O. It is well known that o-Ps decays in H_2O by pick-off annihilation mechanism and there are no any chemical reactions with Ps [18, 22]. Therefore, low value of I_N for Imferon could be explained by the presence of Cl^- ions which are partial inhibitors of Ps formation due to generation of the positron bound states $[e^+, Cl^-]$. On the other hand, the value of I_N for Imferon appeared to be higher than that of 0.9% NaCl solution in spite of the equal Cl^- concentration. It may be related with Ps formation in the free volume holes of the dextran shell due to limited Cl^- accessibility into the shell. The differences of I_N values indicate that p-Ps annihilation in the Imferon and H_2O is essentially distinct from that in 0.9% NaCl solution. The less values of Γ_W for Dextrafer and IDC3 (at $C_{Fe} = 50$ mg/ml) could be attributed to the variations

of the momentum distribution of external electrons which took part in annihilation of quasi-free positrons and o-Ps pick-off annihilation as well as to the different Cl^- permeability into the dextran shell. These changes may be caused by the different molecular weight and structure of dextran. The Fe^{3+} ions of iron core did not react with Ps because in the opposite case an increasing of C_{Fe} in solutions of iron-dextran complexes from 10 to 50 mg/ml should lead to a decrease of I_N and Γ_W values.

As for dissolved iron-dextran solutions ($C_{Fe} = 10$ mg/ml), the value of Γ_W for Imferon coincided with that of 0.9% NaCl solution while parameters of the narrow component were not changed. It could be a result of the increased contribution of $[e^+, Cl^-]$ annihilation to the wide component while the narrow component related with the dextran shell only. The values of Γ_N and Γ_W for Dextrafer had the same tendency as those for Imferon during dilution except for the values of I_N. However, the values of statistical errors for I_N did not permit us to give any reliable conclusions. In contrast to Imferon, the values of I_N and Γ_N for IDC3 coincided with those of 0.9% NaCl solution while the values of Γ_W were different for these samples. This result could be explained by the different molecular weight and structure of dextran and different conditions of iron-dextran preparation as well as possible effects of micelles formation leading to the decrease of annihilation parameters (see [23]). Parameters of the narrow component for IDC1 and Imferon were the same while the value of Γ_W was broader for IDC1. It could be a result of the p-Ps annihilation in the similar free volume holes of Imferon and IDC1 with the same quantity of holes in both iron-dextran complexes. On the other hand, Γ_W value for IDC1 indicated a higher molecular weight of dextran and, therefore, more compact packing of the dextran shell.

Mössbauer Parameters

Unfortunately, we could not give a complete discussion of the Mössbauer results due to unavailable measurements below 87 K. Nevertheless, we could clearly see the main differences between iron-core structures of Imferon, IDC 1–4, and Dextrafer. The α-FeOOH-like particles of Dextrafer did not accumulate in the body. As far as concerning Mössbauer parameters for IDC1 and IDC3, we could not clearly distinguish their iron cores without measurements below 87 K. However, there are different ways of preparing these complexes (in alkaline and acid solutions). The well known Mössbauer data for ferritin showed a poor quadrupole split doublet at 77 K as Mössbauer spectra of IDC1 and IDC3. However, the value of ΔE_Q for ferritin (0.65 mm/s [24]) appeared to be closer to that of paramagnetic component of Imferon than ΔE_Q values of IDC1 and IDC3. Moreover, approximation of ferritin Mössbauer spectra measured at 293 and 77 K with two quadrupole doublets [24] showed ΔE_Q values lower than that given in Table 2. Therefore, these iron-dextran complexes could contain only a ferritin-like iron core with variations.

CONCLUSIONS

Thus, we could see that both Mössbauer and positron annihilation parameters indicated structural differences for studied iron-dextran complexes. It is very important that microstructural variations of the dextran shell and iron core take

place for the complexes with different pathways in the body although we could not yet find direct relations between iron-dextran accumulation and physical parameters.

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