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SELF-ASSOCIATION OF OXYHAEMOGLOBIN. A NUCLEAR MAGNETIC RELAXATION STUDY IN H_2O/D_2O SOLUTIONS.

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The proton and deuterium longitudinal relaxation rates were studied at room temperature up to the highest protein concentrations in oxyhaemoglobin solutions of different H_2O/D_2O composition. The deuterium relaxation rates followed the experimentally well known single linear dependence on protein concentration, the slopes being little influenced by solvent (D_2O/H_2O) composition. The proton relaxation rates show two different linear dependences on haemoglobin concentration. The entire concentration range is described by two straight lines with the threshold concentration about 11 mM (in haem). The ratio of the slopes is 1.6 (high-to-low Hb-conc.). Only in the higher concentration range two T_1's were observed if the solvent contained more than half of D_2O. The slow relaxation phase of protons has T_1's similar to those measured in solutions with less than half of D_2O. The relaxation of the other phase was ten times faster. The ratio of the proton populations in these two phases was equal to 2 (slow-to-fast) and independent of protein concentration. The fast relaxing protons are attributed to water molecules encaged within two or more haemoglobin molecules which associate for times long enough on the PMR time-scale.

1. Introduction

Tomita and Riggs [1] showed a time dependent effect of deuteration on the cooperatively of haemoglobin oxygenation, but it was found by Cupane, Palma and Vitrano [2] that the effect is stable in unbuffered or phosphate-buffered solutions. They studied the degree of cooperativity of haemoglobin oxygenation in dependence on temperature and isotopic (H_2O/D_2O) composition of the solvent. The Hill coefficient, n, which is an overall measure of the cooperativity, was found to be smaller by 0.5 than the ordinary value ($n = 2.9$) above certain $D_2O/(H_2O + D_2O)$ molar ratios and temperatures. This may be due to alteration of the protein hydration. We therefore thought it worthwhile to approach the problem by proton and deuterium magnetic relaxation (PMR, DMR) technique. This, however, required rather highly concentrated haemoglobin solutions as distinct from the aforementioned and other spectroscopic studies. We found it necessary to investigate first in a systematic way the influence of the concentration of haemoglobin upon PMR (and DMR) before the relation of haemoglobin hydration and cooperativity could be dealt with directly.

A survey of the literature showed that high haemoglobin concentration may be also of physiological importance. For instance, Rossi-Fanelli, Wyman, Antonini and Caputo [3] found a slight dependence of the half-saturation oxygen tension ($p_{1/2}$) in solutions below 1 mM. That study was extended by Redford, Torelli, Celentano and D'Angelo [4,5] to the concentrations up to some 5 mM, i.e. to the highest attainableones, like in erythrocytes. A 50% increase in $p_{1/2}$ was detected, but no change in subunit cooperativity. The concentration dependence of $p_{1/2}$ for haemoglobin solutions has been discussed by Benesch, Benesch and Yu [6]. Information about further physiological consequences related to the allosteric effectors can be found in the book of Alfred Benzon Symposium IV, and in particular in the papers by Rapoport, Gerber, Ruckpaul, Jänig, Frunder and Jung [7], and by Lenfant, Bellingham and Detter [8]. Another very important point of physiological significance in this respect is the osmotic pressure behaviour of red cells, which was extensively dealt with by

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Solomon and coworkers (for a survey see ref. [9]).

In this paper we discuss the PMR evidence in the light of association of haemoglobin molecules above certain threshold concentration in solutions of oxyhaemoglobin.

2. Materials and methods

2.1. Haemoglobin and solvents

Oxyhaemoglobin was prepared from fresh human blood, following the method of Cameron and George [10]. After dialysis against deionised water the oxyhaemoglobin solution was dialysed against 0.1 M phosphate pH 7 (39.0 ml KH_2PO_4 and 53.6 ml K_2HPO_4 onto 1000 ml). The solution was then concentrated by pressure dialysis in a visking bag of 8 mm diam. applying about one atm. of nitrogen gas. The concentrated haemoglobin solution was further diluted by 0.1 M phosphate in D_2O, (Prochem B.O.C., 99.8%) measured pH 7.1, in one to one volume ratio. The procedure of concentrating and diluting the haemoglobin solution with deuterated buffer was repeated four times allowing more than 12 hours for the H–D exchange between each step. Altogether the H–D exchange lasted approximately 5 days (at 4°C) and in view of the permanent effect of exchange in phosphate-buffered solutions [2] it is expected that all exchangeable protons from the protein were exchanged for deuterium.

Solutions of desired H_2O/D_2O ratio were finally obtained by diluting with H_2O-phosphate buffer. These solutions were approximately 10 mM in haem. Each of them was devided in two parts, one of which was concentrated up to 20 mM per haem. The two stock solutions were centrifuged at 12000 g for half an hour before mixing them in differing proportions to obtain a set of Hb-concentrations at a constant H_2O/D_2O ratio. The lower concentrations (<10 mM) were obtained by diluting with corresponding H_2O/D_2O buffer. The procedure for obtaining the more concentrated set of solutions was found necessary in order to avoid partial denaturation observed when direct dilution with solvent was tried. The control T_1-values from (H_2O/D_2O) solvents and from that after the final concentration of the stock solutions were equal. All the measurements were completed in less than two days from the final adjustment of both D_2O and Hb concentration.

The concentrations of all solutions were determined using the cyanmet-method [11]. The spectra in the visible range which were taken for determining the content of methaemoglobin in oxyhaemoglobin solutions, showed less than 5% of metHb in all samples.

2.2. T_1-measurements

The deuterium and proton longitudinal relaxation times, T_1, were measured by the 180°-t-90° pulse sequence. The digital readout pulsed spectrometer (Institute "Jožef Stefan" Ljubljana) with a high resolution magnet (Bruker-Karlsruhe) was used at 12 MHz for both nuclei and at 24 MHz for protons.

A computer program yielded T_1-values for deuterium usually with a correlation coefficient better than 0.99 for the least-squares straight lines. For the resolved T_1's of protons (see later) it was not less than 0.99 for *S*-phase protons, and about 0.97 for *F*-phase protons. Maximal errors of T_1 were about 5% for deuterons and *S*-phase protons and about 9% for *F*-phase protons.

The temperature of the samples was kept constant at 24°C within ±1° by a thermostated nitrogen stream. The actual temperatures of solutions were measured directly with a thermocouple before and after T_1-measurements.

3. Results

The fundamental difference between the relaxation mechanism of protons and deuterons is caused by the fact that protons relax mainly by the magnetic dipole–dipole interaction while the dominant relaxation mechanism for deuterons is the fluctuation of the electric field gradient at the deuteron [12]. It is therefore expected that the proton relaxation rates will be more sensitive to isotopic dilution (H_2O/D_2O) than those of deuterium. Relevant measurements in degassed pure water have been reported by Anderson and Arnold [13]. For practical purposes of the present study we needed such information on the actual solvent used, i.e. air-saturated water containing 0.1 mM phosphate pH 7.0. The results are given in fig. 1 and will be used in calculating the relaxation rates induced by the

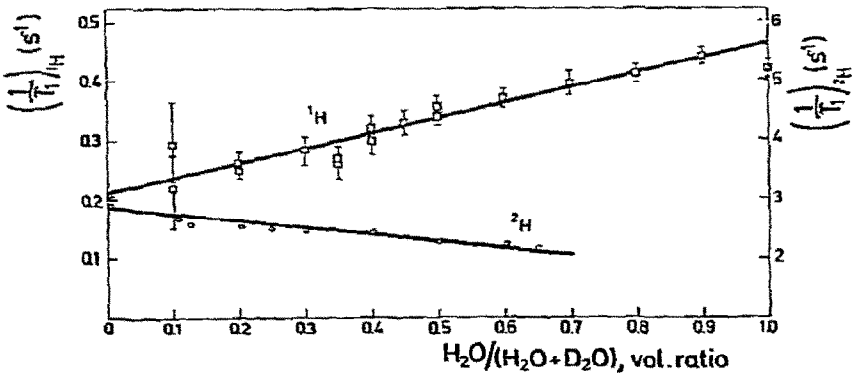

Fig. 1. The longitudinal relaxation rates, $1/T_1$, of protons (□, ordinate scale on the left) and deuterons (○, ordinate scale on the right) in the dependence on the H_2O-content of H_2O/D_2O mixed solvent, 0.1 M phosphate, pH 7, air-saturated at 23°C. The straight lines are least-squares best-fits.

presence of protein in solutions of varying H_2O/D_2O-composition.

Fig. 2 shows the dependence of deuterium relaxation rates on oxyhaemoglobin concentration in solvents of four different H_2O/D_2O compositions. Table 1 summarizes the straight-line regression data for each of the four solvents. The regression coefficient is generally quite good, being less than 0.99_3 only for the solution with least of deuterium in the solvent. The lower regression coefficient for this 0.65 H_2O-solution is paralleled with the substantially larger error ±0.4, compared with ±0.2 to 0.3 for the other three solutions. This may be ascribed to the dependence of the measurement sensitivity on deuterium concentration. Taken altogether the data from table 1 indicate that DMR rates depend more sharply on haemoglobin concentration in solvents with more deuterium, but that a single straight line dependence holds throughout the concentration range up to the maximal concentrations of haemoglobin for each particular solvent.

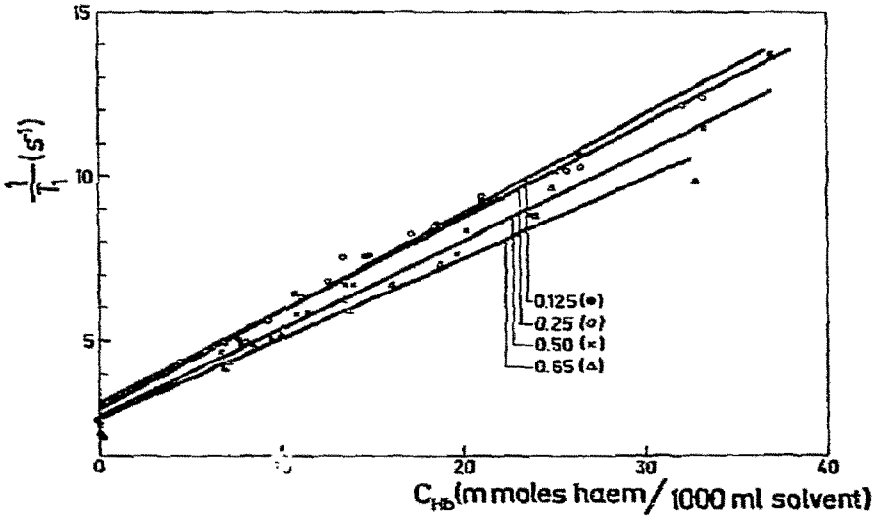

Fig. 2. The longitudinal ^2H-relaxation rates, $1/T_1$, at 12 MHz and 24°C in dependence on oxyhaemoglobin concentration in four solutions of different $H_2O/(D_2O + H_2O)$-volume ratio as indicated. The straight lines are least-squares best-fits for each solution (see table 1).

Table 1
Straight-lines regression parameters for data in fig. 2

αH_2O	The measured values			
	$1/T_1$ =	regr. coeff.	$\Delta 1/T_1 = 1/T_1 - (1/T_1)_{solv.}$ =	regr. coeff.
0.125	$(0.299c + 2.90) \pm 0.25_1$	0.99_7	$(0.308c + 0.57) \pm 0.20_7$	0.99_7
0.25	$(0.282c + 3.10) \pm 0.31_0$	0.99_5	$(0.296c + 0.72) \pm 0.28_3$	0.99_4
0.50	$(0.269c + 2.70) \pm 0.28_4$	0.99_4	$(0.278c + 0.73) \pm 0.25_5$	0.99_2
0.65	$(0.243c + 2.65) \pm 0.40_4$	0.98_3	$(0.252c + 0.76) \pm 0.39_6$	0.98_2
all			$(0.288c + 0.61) \pm 0.45_6$	0.98_4

α_{H_2O} = H_2O volume/solvent volume; c = mmoles haem/1000 ml solvent.

While the deuterium magnetization had a single straight-line course in the semilog plots, those of proton magnetic longitudinal relaxation indicated two relaxation times for solutions of higher haemoglobin concentrations in solvents containing less than half of H_2O. All the PMR data were subsequently subjected to an iterative computer procedure to see if the originally measured magnetization curves could be resolved into two independent ones yielding two different T_1's and the fraction of each of the two relaxing species ($S + F = 1$). Two such examples are shown in fig. 3; one is with rather high protein concentration ($C = 25.5$ millimoles haem per 1000 ml solvent) and small content of protons ($\alpha = 0.25$), so that the graphical analysis is obvious; the other was chosen as a borderline case, i.e. at the threshold concentration (~13 millimoles haem per 1000 ml of solvent, equal to 11.5 mM in haem) and at the highest H_2O content at which two T_1's were still discernible. In no case for solutions with $\alpha > 0.5$ and below haem-concentration of 10.5 mM were we able to obtain two magnetization curves from the experimental data. In those cases (higher Hb-concentrations and lower H_2O content) where two relaxation times were discernible we term the long ones S (for slow relaxation phase), and the shorter ones F (fast phase). Though somewhat shorter, the long times are still

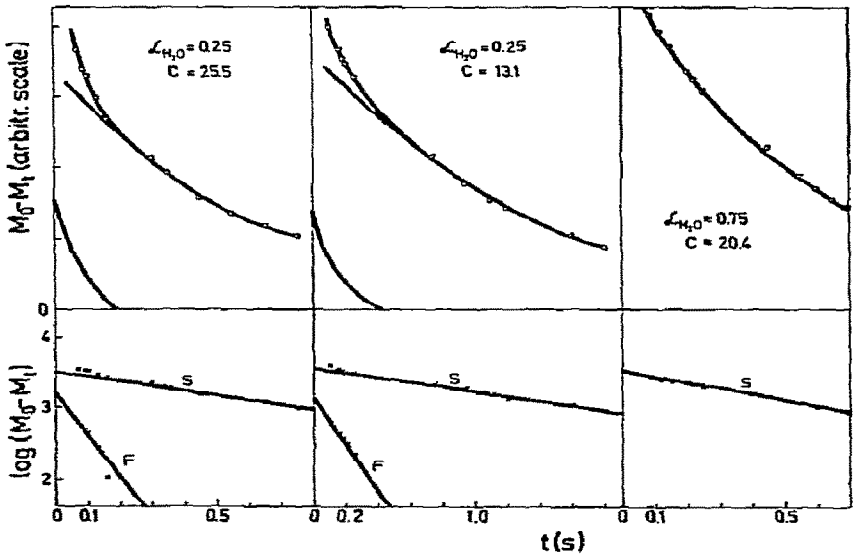

Fig. 3. Examples of magnetization curves for oxyhaemoglobin solutions of different protein- and H_2O-composition, measured at 24 MHz, 24°C. $\alpha_{H_2O} = H_2O/(H_2O + D_2O)$; c = mmol haem/1000 ml solvent.

Table 2
The measured relaxation rates, $1/T_1(s^{-1})$, for solutions of different isotopic (H_2O/D_2O) composition in dependence on oxyhaemoglobin concentration

12 MHz								24 MHz							
$\alpha_{H_2O} = 0.125$		$\alpha_{H_2O} = 0.25$		$\alpha_{H_2O} = 0.50$		$\alpha_{H_2O} = 0.65$		$\alpha_{H_2O} = 0.15$		$\alpha_{H_2O} = 0.25$		$\alpha_{H_2O} = 0.50$		$\alpha_{H_2O} = 0.75$	
c	$1/T_1$	c	$1/T_1$	c	$1/T_1$	c	$1/T_1$	c	$1/T_1$	c	$1/T_1$	c	$1/T_1$	c	$1/T_1$
0.00	0.23	0.00	0.31	0.00	0.39	0.00	0.40	0.00	0.22	0.00	0.26	0.00	0.36	0.00	0.40
5.18	0.72	2.43	0.46	7.45	1.05	5.99	0.82	2.03	0.23	1.99	0.35	3.95	0.47	2.35	0.44
7.25	0.87	3.20	0.56	9.68	1.15	7.58	0.97	2.79	0.34	4.40	0.37	4.67	0.48	3.16	0.49
9.13	1.29	6.18	0.84	10.65	1.28	8.79	1.24	6.43	0.45	6.04	0.46	4.90	0.48	3.31	0.46
11.03	1.34	7.22	1.02	11.21	1.38	11.19	1.08	7.61	0.56	6.99	0.48	6.46	0.51	4.77	0.52
17.26	1.62	9.73	1.09	15.10	1.66	11.52	1.24	8.30	0.55	7.43	0.51	7.11	0.57	7.20	0.63
17.26	1.53	11.05	1.30	16.61	1.83	13.38	1.37	13.15	0.89	8.18	0.58	11.60	0.97	7.92	0.79
19.19	1.91	13.30	1.66	17.61	2.04	14.07	1.56	16.38	1.03	13.00	0.86	14.38	1.01	9.64	0.88
20.01	1.95	16.42	1.77	18.58	2.42	15.44	1.25	23.45	1.24	15.09	0.97	16.42	1.23	11.36	1.00
26.32	2.59	18.44	1.83	21.74	2.80	17.48	1.86	25.68	1.61	16.29	1.06	18.16	1.50	11.42	0.87
26.49	3.51	20.39	2.26			21.53	2.54	34.24	2.55	19.73	1.22	20.01	1.41	16.96	1.23
34.60	4.64	22.68	3.00			26.66	3.04			21.20	1.38	23.25	1.91	20.36	1.75
						30.80	3.46			22.67	1.61	27.89	2.36		
										28.01	2.09	31.20	2.70		

comparable to those for the low Hb-concentration range, while the *F*-phase has relaxation times 10 times shorter.

In table 2 we present all the original experimental PMR data for the *S*-phase which is the only one for lower Hb concentrations irrespective of the solvent H_2O/D_2O-composition, and for all Hb concentrations if $\alpha > 0.5$, measured at two Larmor frequencies (12 and 24 MHz). These data were analysed as to the best fit using a programme for curves with increasing degrees. The result of this analysis is presented in table 3. The conclusion is that the "best-fit" with one single straight-line for *all* the points is the worst. The goodness of the best fit appears to level off for curves of the third and fourth degree. We matched these data to two straight lines, one for the lower, the other for the higher concentration range. In doing so the original values were corrected for the solvent contribution (fig. 1) to the relaxation rates taking into account the volume occupied by haemoglobin. The final result is presented first graphically in fig. 4. The lower concentration range is represented well by a single common straight line, i.e. the slope of the concentration dependence of the relaxation rates does not depend on solvent (H_2O/D_2O) composition. For the higher concentrations of haemoglobin there are two straight lines, both different from that for the lower concentrations. The significance of the difference between the two high-concentration lines

Table 3
The values $\Sigma(\Delta y)^2$ for the data in table 2

n	12 MHz				24 MHz			
	α_{H_2O}				α_{H_2O}			
	0.125	0.25	0.50	0.65	0.15	0.25	0.50	0.75
1	1.522	0.343	0.228	0.503	0.238	0.140	0.254	0.098
2	0.717	0.242	0.086	0.293	0.084	0.017	0.053	0.047
3	0.579	0.156	0.059	0.288	0.046	0.017	0.044	0.046
4	0.504	0.065	0.052	0.209	0.030	0.016	0.044	0.020

$\Sigma(\Delta y)^2 = \Sigma(y_t - y_e)^2$; y_t is a value of ordinate calculated from the best fit; y_e is an experimental value of the same ordinate; n is the degree of the fitting equation.

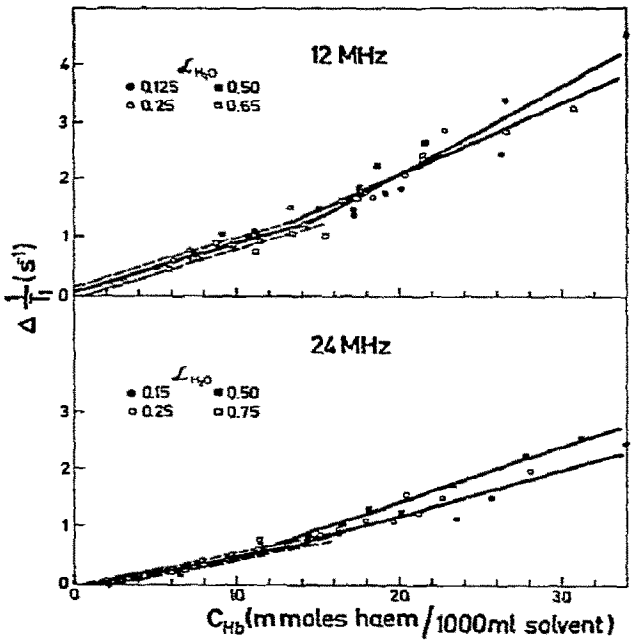

Fig. 4. The longitudinal magnetic relaxation rates of the *S*-phase protons in the dependence on the oxyhaemoglobin concentration for solutions of different α_{H_2O}, as indicated, at 24°C and two Larmor-frequencies (12 and 24 MHz). The parameters of the straight-lines best-fits are given in table 4.

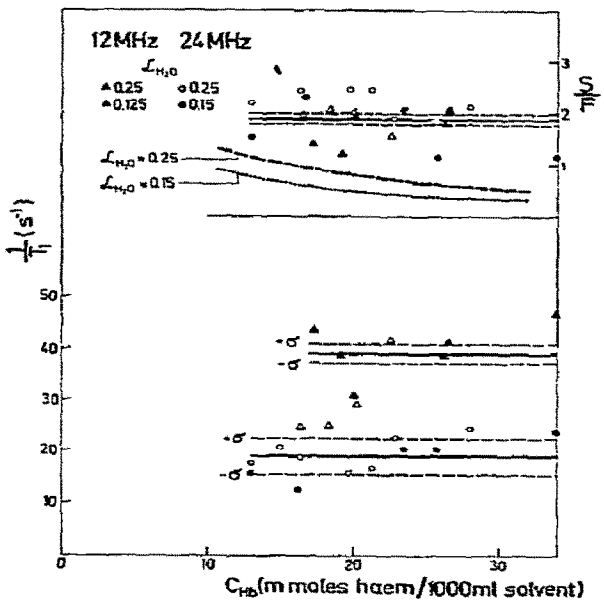

Fig. 5. Lower part: the longitudinal proton magnetic relaxation rates of the fast-relaxing phase(F), in the dependence on oxyhaemoglobin concentration for solvents of various α_{H_2O} (as in fig. 4). Upper part: the ratio of the proton-abundance in the slow(S) – to that in the fast(F) – relaxing phase. For doted lines see section 4.

Table 4
Straight-lines regression parameters for data in fig. 4

αH_2O	Range of haemoglobin concentrations					
	low		high			
	$1/T_1$ =	regr. coeff.	$1/T$ =	regr. coeff.	$1/T$ =	regr. coeff.
12 MHz						
0.125	$(0.120c-0.155) \pm 0.07_4$	0.96_7	$(0.175c-1.630) \pm 0.25_1$	0.97_3 }	$(0.155c-1.040) \pm 0.29_0$	0.94_5
0.25	$(0.096c-0.017) \pm 0.05_1$	0.98_6	$(0.138c-0.565) \pm 0.22_0$	0.90_0		
0.50	$(0.086c+0.076) \pm 0.03_8$	0.95_6	$(0.184c-1.345) \pm 0.08_5$	0.97_9 }	$(0.126c-0.450) \pm 0.22_0$	0.94_0
0.65	$(0.078c-0.041) \pm 0.10_0$	0.90_8	$(0.138c-0.860) \pm 0.16_3$	0.97_9		
all	$(0.084c+0.050) \pm 0.10_4$	0.92_6				
24 MHz						
0.15	$(0.059c-0.136) \pm 0.02_1$	0.99_7	$(0.078c-0.436) \pm 0.17_2$	0.95_8 }	$(0.078c-0.394) \pm 0.13_2$	0.96_5
0.25	$(0.051c-0.089) \pm 0.03_8$	0.97_4	$(0.083c-0.465) \pm 0.06_8$	0.98_6		
0.50	$(0.065c-0.152) \pm 0.04_9$	0.98_6	$(0.101c-0.640) \pm 0.06_9$	0.99_2 }	$(0.094c-0.481) \pm 0.09_1$	0.98_7
0.75	$(0.063c-0.167) \pm 0.03_9$	0.97_2	$(0.087c-0.325) \pm 0.09_9$	0.95_9		
all	$(0.057c-0.099) \pm 0.04_8$	0.97_1				

$\alpha H_2O = H_2O/(D_2O + H_2O)$, volume ratio; c = mmoles haem/1000 ml solvent; $1/T_1$ = relaxation rate in s^{-1}.

Table 5
Relaxation times and relaxation rates of protein protons in deuterated oxyhaemoglobin solutions, measured at 24 MHz

c (mM)	t (°C)	T_1 (ms)	$1/T_1$ (s^{-1})
5.36	24	157 ± 7	6.36
9.86	24	150 ± 1	6.66
15.80	24	120 ± 1	8.30
15.80	29.5	136 ± 3	7.35
15.80	16	148 ± 2	6.76
		grand mean	7.08 ± 0.34

is not well established. For instance, at 24 MHz the smaller slope is that for $\alpha_{H_2O} = 0.15$ and 0.25 solutions while at 12 MHz the reverse is the case. In view of the greater uncertainty in this concentration range, possibly owing to concentration errors it is not unlikely that also for the higher concentrations only one straight line may be real. The relevant numerical data as to the straight-lines fits are given in table 4.

As mentioned above, the higher concentration range had two relaxation phases: *S* (dealt with in fig. 4 and table 4), and *F*, which is characterized by fig. 5. The lower half of fig. 5 contains the PMR rates for the "fast" *F*-phase. These data are independent of haemoglobin concentration, with a mean value for $1/T_1 = 18.8 \pm 3.5\ s^{-1}$ measured at 24 MHz. In calculating the mean value for the rates at 12 MHz, $38.9 \pm 1.8\ s^{-1}$, only the points obtained with $\alpha_{H_2O} = 0.125$ were taken into account, as the others (for $\alpha_{H_2O} = 0.25$ solvent) did not appear to be certain enough. The upper half of this figure summarizes the values for the ratio of the *S*(slow)-to-*F*(fast) relaxation phases. This ratio seems to depend neither on Hb concentration nor solvent composition, the grand-mean value being 1.88 ± 0.09.

The ratio of the relaxation rates at 12 MHz to those at 24 MHz is 1.7 for *S*-phase (fig. 4) and 2.1 for *F*-phase (fig. 5) without the data for $\alpha_{H_2O} = 0.25$, and 1.9 if all the data (for *F*) are taken into account.

Table 5 comprises PMR data obtained with fully deuterated oxyhaemoglobin solutions. All the magnetization curves were monophasic, indicating that the measured values are due solely to the protons from the protein.

4. Discussion

4.1. The deuterium relaxation in D_2O/H_2O Hb-solutions

The DMR results from fig. 2 are in agreement with the well known linear dependence on protein concentration [14], except that we extended substantially the concentration range. The differences in slopes of the straight lines corresponding to the four different solvent compositions (table 1) are not dramatic. They do show a definite trend towards larger slopes for increasing deuterium content of the solvent. Glasel [15] pointed out that because of its magnetogyric ratio, deuterium is more convenient (^{17}O even more so) than protons for sensing the (hydration) interaction of water molecules with the dissolved protein. However, from the point of view of the main problem, that of the relationship between hydration and oxygenation cooperativity [2], it remains to be seen what information as to the hydration structure can be obtained from DMR data in dependence on temperature [16]. Besides, a reviewer of this paper suggested that notwithstanding the magnetogyric ratio ^2H is very *in*convenient for sensing the water interaction with the protein. Exchange of ^2H with for instance COOD groups may lead to a domination of the overall relaxation rate by the relaxation in the protein-bound sites.

4.2. The proton relaxation in D_2O/H_2O Hb-solutions

4.2.1. Two relaxation phases – the dependence on solvent composition

The PMR results (figs. 4 and 5 and table 4) appear to be more rewarding. The first finding is that of the two relaxation phases, slow (*S*) and fast (*F*). The slow one is quite common, both in respect to the absolute values of the protein-induced relaxation rates and their linear dependence on haemoglobin concentration [14,18]. The quoted references include measurements mainly in the lower concentration range and hence no kinks in the linear dependence were noted. It seems reasonable that the second straight line is in the concentration region where the double exponential relaxation is beginning to be felt (in solutions with less than 50 vol% H_2O).

The "ordinary", slow phase (*S*) is due to the interaction of water molecules with the protein surface implying that most if not all of these molecules are in

rapid exchange (on the NMR time scale) with those from the bulk solvent [19,20]. Hence, *one* phase in the NMR sense. Such is the case even for the high-concentration range if there is enough (more than 50%) protons in the solvent. It is reassuring that, on the other hand, when the computer procedure distinguished between two (relaxation) phases (figs. 3, 4 and 5) the derived slow phase (for $\alpha \leqslant 0.25$) was also commensurable in its protein-concentration dependence and in absolute values with the PMR rates obtained when only the *S*-phase was observed (for $\alpha > 0.5$). The detection of the fast, *F*-phase, means that, on the NMR time scale, there are solvent-molecules without rapid, or efficient enough exchange with the bulk solvent.

The lack of the fast phase above the threshold haemoglobin concentration in solvent with more than half of ordinary water may be rationalized as follows.

It is unlikely that the *F*-phase relaxation mechanism depends on the proton content of the solvent as to become faster with increased α. In such a hypothetical case, for which the *F*-phase relaxation could not be measured because it would be too fast, the experimentally determined total magnetization, comprising all the protons ($M_{0\,\mathrm{exp}}$) must be larger than that calculated from the best-fit exponential ($M_{0\,\mathrm{cal}}$). As we have $S/F \approx 2$ (fig. 5), $M_{0\,\mathrm{exp}}/M_{0\,\mathrm{cal}} = (S+F)/S = 1 + F/S \approx 1.5$, while from all our actual data $M_{0\,\mathrm{exp}}/M_{0\,\mathrm{cal}}$ was 1.2 to 1.1. The latter values are fully accounted for by the neglected fast-relaxing protons from the protein.

The two phases (*F* and *S*) may be observed if the exchange of protons between them is too slow to average out the corresponding relaxation times. Assuming the dominant exchange mechanism is via water molecules or hydronium ions, its rate will not be affected substantially by isotopic dilution. However, for $\alpha < 0.25$ most of the exchanging molecules will be without any protons and some with only one. With $\alpha > 0.5$ there will be a great number of molecules with even two protons. Hence, although the actual rate of exchange may not be altered, an increase in proton-content should result in a much more efficient magnetic coupling between the *F*- and *S*-phase, which may eventually cause an averaging out of the two relaxation rates. But then the *S*-phase relaxation rate for $\alpha > 0.5$ must be larger than for $\alpha < 0.25$ (when the *F*-phase is observable separately). An indication of this effect may be found in the data of fig. 4, but much more elaborate measurements are required to reach a firm conclusion.

4.2.2. The origin of the fast-relaxing phase (F)

The nonexistence of phase-*F* in solutions below the critical (10.5 mM) haemoglobin concentration must have something to do with the state of dissolved protein, and not only with the solvent and its relaxation mechanism.

We shall first consider the possibility that the fast relaxation is due solely to the protons from the protein (haemoglobin).

On average, there are 1130 protons per each haemoglobin subunit. Of them all about 240 are exchanged for deuterium if kept for over a day in a predominantly D_2O-containing solvent [21] like it was in the present experiments. The dotted lines in fig. 5 indicate the calculated concentration-dependence (for $\alpha = 0.15$ and 0.25) of the ratio of the *S*-phase (liquid) to the *F*-phase, if the latter is assumed to be only from the nonexchangeable protons of the dissolved haemoglobin. Such a dependence is not born out experimentally, the measured ratio S/F being 1.88 ± 0.09, but more detailed studies at different frequencies are still desirable.

Further, the relaxation rates of protons from fully deuterated oxyhaemoglobin in D_2O-solutions (see table 5) are significantly smaller than those of the *F*-phase in fig. 5 (for 24 MHz). It is therefore unlikely that the *F*-phase is due to the protons from the protein molecules. Much more probable is the other alternative, that the fast relaxation originates from *solvent* protons "isolated" on the PMR time-scale from those with the slow relaxation rate.

Consistent with this notion is the fact that the threshold haemoglobin concentration, at which two relaxation phases become observable (when $\alpha < 0.5$), does not depend on the solvent D_2O/H_2O composition. If only the protons from the protein were contributing to the *F*-phase, the detection of their fast relaxation should depend on the H_2O content of the solvent. In other words, the lower the H_2O-content the lower ought to be the threshold haemoglobin concentration, which is not observed. The *F*-phase is detectable in a 25 vol% H_2O solvent but not if there is 50 vol% H_2O. This cannot be explained by the *F*-phase being due to the protein-protons, because the high haemoglobin-concentration range was sufficient (factor of two) to compensate the twofold increase in the content of *S*-protons.

4.2.3. The two-phase molecular model (frequency dependence of the relaxation)

The characteristics of the fast *F*-phase are as follows.

(a) The protons belonging to this phase relax faster then those of the fully ^2H exchanged protein in D_2O (see fig. 5 and table 5); (b) their amount relative to those of the slow *S*-phase, and (c) their relaxation rate (fig. 5) may not depend on the protein concentration (as does the rate of the protons in the slow, *S*-phase); finally, (d) the protons from the *F*-phase are *not* those from the protein, *though intimately connected* with it, but magnetically "isolated" from the bulk solvent.

We therefore come to the conclusion that the *F*-phase could only be envisaged as part of the solvent encaged by haemoglobin molecules. The implicit assumption in the last conclusion is that haemoglobin molecules associate for times long enough to form the structured, encaged water phase, observable by a double exponential decay of the total longitudinal magnetization. The concentration of haemoglobin at which such macromolecular association may take place is related to the free mean-path for the diffusing haemoglobin molecules. A complicated equilibrium in terms of dynamics and size of the "aggregates" may be expected above the threshold protein concentration. A simple case would be such interaction of Hb-molecules that their central cavities are blocked. Some 300 H_2O-molecules per $[\alpha\beta]_2$ could therefore be magnetically uncoupled from bulk solvent for the life-time of the "aggregates", while the specific ("outer") hydration in phase-*S* would remain practically constant, so that the ratio *F/S* may also be independent of protein concentration as appears to be born out experimentally. It was shown recently by Koenig, Hallenga and Shporer [22] that no direct relation may be proved between the *amount* of protein hydration and relaxation rates. Their general conclusion was that the NMR-correlation time of the water molecules under the influence of the protein molecule *will* by and large reflect the rotational tumbling time of the latter, and hence the size of the macromolecule.

The PMR-rates at two frequencies (12 and 24 MHz) given in fig. 4 are different, indicating that the pertinent "effective" correlation time, τ_c, must be $>10^{-9}$ s as can be seen from the corresponding correlation functions in fig. 6. Now, the low-concentration *S*-phase relaxation measured at the two frequencies yields for the respective rates a ratio of 1.87, while the high-concentration ratio is about 1.63. This means that the "effective" correlation time is close to 4×10^{-9} s (see fig. 6). The ratio of the straight-line slopes for the

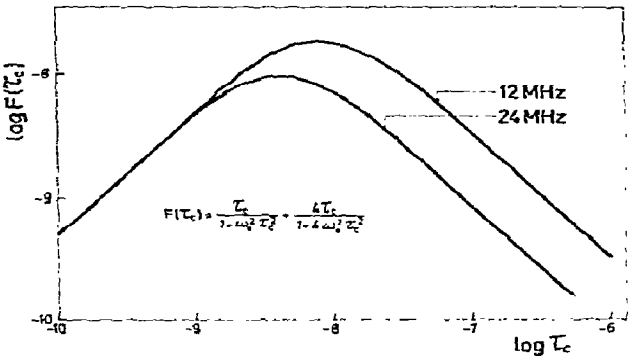

Fig. 6. The correlation function $F(\tau_c)$ for two frequencies 12 MHz and 24 MHz.

high-to-low concentration region (in fig. 4) is about 1.6 (for either frequency). This (1.6 ×) increase in relaxation vs. concentration slope could be explained by an increase in τ_c (due to viscosity for instance) *only* if the ascending part of the correlation curves in fig. 6 were involved. This is not the case, the initial (low-concentration) τ_c being just at about the maxima of the curves. The relaxation rate is proportional to the correlation function, $F(\tau_c)$, and to the amount of the rotationaly hindered water molecules (of hydration) [14,23]. With the longer τ_c for the higher concentration range $F(\tau_c)$ at 12 MHz would not change and it would even slightly diminish at 24 MHz. Hence, the increased slope (by 1.6 ×) would mean that it is due entirely to a slightly increased amount of hydration, which in turn, can also be rationalized by association of $(\alpha\beta)_2$-haemoglobin molecules.

The relaxation rate of the *F*-phase doubles on going from $\nu_L = 24$ to $\nu_L = 12$ MHz. This implies that correlation times longer than 10^{-8} s must be involved as derived from fig. 6. On the other hand the absolute rates of the fast phase (*F*) are about 10 times greater than for the *S*-phase (compare figs. 4 and 5). It is out of question that such an enhanced rate can be due to a longer tumbling time of the aggregates because the same rate or tumbling pertains to both relaxation phases (*S* and *F*). An even more efficient relaxation mechanism perhaps must be sought for. Further temperature and frequency studies are required for elucidation of the relaxation mechanism and the hydration structure in phase *F*.

The present picture is as follows. Below some 11 mM

per haem solutions of Hb consist of individual Hb-molecules surrounded by an "open" solvation sheath from which the water molecules are in fast exchange with bulk solvent forming thus a single-relaxation phase on the PMR time scale. The solution relaxation rates are simply linearly dependent on Hb-concentration because of the favourable slowing down of the H_2O-tumbling rate due to the interactions with the surface of individual protein molecules.

Above the threshold protein concentration haemoglobin molecules associate, with aggregates of lifetimes longer than the longest relaxation time(s) in their "open" solvation sheath. This hydration is somewhat increased (by a factor of about 1.6) compared to the low-concentration solution state. Within these (dynamic) aggregates an additional, "closed" hydration exists, such that the water protons from it are magnetically uncoupled with respect to the "open" solvation in the bulk solvent if the latter is isotopically sufficiently dilute.

The dominant relaxation mechanism within the "closed" hydration is unknown. It is more effective not only than the tumbling-mechanism of the "open" hydration, but also than that exibited by the protons of the protein.

The results of the present study by an independent technique, kinetic in nature, are in full agreement with the time-average recording of the low-angle X-ray scattering interpreted [24] by association of $[\alpha\beta]_2$-haemoglobin molecules in concentrated solutions. The possible significance of this finding may not be of interest only with regard to Hb-function indicated in the Introduction, but also in that broader sense of self-associative systems in biology. Other globular proteins may show similar behaviour.

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