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PHOSPHORUS-31 RELAXATION TIMES OF 2,3-DIPHOSPHOGLYCERATE IN INTACT HUMAN ERYTHROCYTES

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SUMMARY: The reciprocals of the spin-lattice relaxation times (T_1s) of the 2-P and 3-P nuclei of 2,3-diphosphoglycerate (DPG) increased linearly as percent DPG bound was raised in model hemoglobin solutions. The 2-P T_1 was slightly greater in intact erythrocytes than in model solutions under similar experimental conditions. The change in the 3-P T_1 with cellular deoxygenation was anomalous indicating that this nucleus should not be used to estimate DPG binding inside intact erythrocytes.

We have previously estimated the percent of bound DPG inside human erythrocytes noninvasively from chemical shifts recorded directly from these cells, using a standard curve constructed from chemical shift and binding data derived from model hemoglobin solutions (1,2). In both model solutions and cells, only one set of DPG signals was seen indicating that there was rapid exchange of ^{31}P nuclei between free and bound forms, with the resonance position reflecting the percentage of bound (or free) species present. In the present communication, we report results from studies in which T_1s of the ^{31}P nuclei of DPG have been determined in intact erythrocytes. This parameter should also reflect the distribution of free and bound forms (see below) and could offer certain advantages in binding studies since it is more directly affected by interactions between molecules.

METHODS

Cation impurities were removed from solutions of DPG and ATP (Sigma) with a Chelex 100 column, 50-100 mesh (Bio-Rad). Hemoglobin was purified by the

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Table 1

Spin-lattice relaxation times (T_1s) of the phosphorus nuclei of 2,3-diphosphoglycerate (DPG).

Expt. No.	System	pH*	Hemoglobin		DPG Conc.	T_1 of DPG**		DPG Bound
			Prelim. Gas Treat.	Conc. mM	mM	sec 3-P	sec 2-P	%
1	Standard DPG Solution	7.20	--	--	5.43	5.41 ± .01	3.63 ± .04	--
2	Model Solution A	7.23	air	2.63	4.62	1.65 ± .04	1.35 ± .03	34
		7.26	N_2	2.63	4.62	1.51 ± .01	1.27 ± .01	44
3	" "	7.22	air	2.74	2.64	1.52 ± .03	1.27 ± .02	43
		7.30	N_2	2.74	2.64	1.03 ± .02	0.94 ± .02	61
4	Model Solution B***	7.17	air	3.12	3.90	1.74 ± .13	1.74 ± .07	37
		7.22	N_2	3.12	3.90	1.47 ± .03	1.40 ± .02	55
5	Intact Erythrocytes	7.15	none	--	--	1.36 ± .28	2.41 ± .16	--
		7.21	N_2	--	--	1.99 ± .10	1.83 ± .11	--
6	" "	7.28	none	--	--	2.36 ± .86	2.89 ± .58	--
		7.20	N_2	--	--	2.56 ± .21	1.84 ± .17	--
7	" "	7.24	none	--	--	1.94 ± .10	2.41 ± .14	--
		7.09	N_2	--	--	2.37 ± .14	1.84 ± .17	--

*Medium pH is shown in Expts. 1-4. Cell pH was estimated from the P_i chemical shift (11) in Expts. 5-7.
**T_1 values are means ± S.E.M. from at least 9 data points. Experiments were conducted at 20° C.
***Model Solution B contained 0.95 mM ATP and 2.22 mM Mg^{2+} in addition to DPG and hemoglobin.

method of Drabkin (3). Model solutions were prepared by dissolving DPG and other solutes in 0.05 M bis Tris/0.12 M KCl/0.03 M NaCl buffer as described in Table 1. Blood was collected in citrate-phosphate-dextrose medium (4) and packed erythrocytes were obtained by centrifuging the blood at 800 g for 10 min. Deoxygenation was effected by shaking a sample in a Dubnoff incubator for 45 min in the presence of a constant N_2 flow.

Ten ml of sample was added to a 20 mm NMR tube which was placed in a wide bore Nicolet NTC-150 spectrometer equipped with a Nicolet 1180 computer. T_1 values were determined on decoupled ^{31}P signals using a fast inversion-recovery pulse sequence (5). A Nicolet three parameter T_1 program was used to fit the intensity data for at least 9 τ values. Procedures for evaluating DPG concentration and percent DPG bound have been described earlier (1).

RESULTS AND DISCUSSION

T_1 values of a standard DPG solution (Expt. 1, Table 1) were similar to values reported by Gupta et al. (6). The T_1 of the 3-P nucleus was slightly

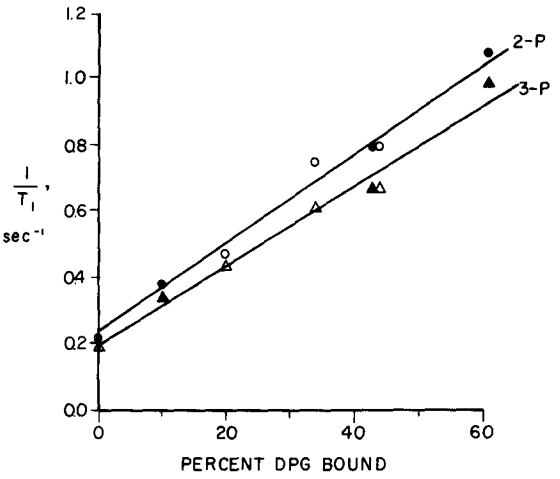

Fig. 1. Reciprocals of spin-lattice relaxation times (T_1s) of the 2-P and 3-P nuclei of 2,3-diphosphoglycerate plotted against percent 2,3-diphosphoglycerate bound in model hemoglobin systems. Open and closed symbols refer to aerated and deoxygenated systems, respectively. The equations of the 2-P and 3-P lines are y = 0.0135x + 0.228 and y = 0.01119x + 0.191. The points on the y-axis are from a standard DPG solution containing no hemoglobin.

greater than that of the 2-P nucleus, which may be due to the fewer number of interactions that the 3-P nucleus experiences within its immediate intramolecular environment. In a solution containing DPG and hemoglobin (Model Solution A, Expt. 2), T_1 values were lower than in the standard solution, due presumably to a fraction of the DPG being bound to hemoglobin (as shown in the last column) causing these nuclei to relax at a more rapid rate*. After deoxygenation, which was associated with greater DPG binding (44%) than in the sample equilibrated with air (34%), further T_1 decreases were observed as were also noted by Gupta *et al* (6). In Expt. 3, a lower DPG concentration was used to increase the percent DPG bound and T_1 values were all smaller than the corresponding values in Expt. 2. When T_1s obtained at various levels of DPG binding were plotted as their reciprocals (*i.e.* relaxation rates) against

*The T_1 of bound DPG in DPG-hemoglobin solutions can be calculated from an equation of the relaxation rate, $\frac{1}{T_s} = \frac{x}{T_b} + \frac{1-x}{T_f}$, where T_s, T_b, and T_f are T_1s of the DPG-hemoglobin solution, bound DPG and free DPG, respectively, and x is the fraction bound. Using the values obtained in Expts. 1 and 3, T_b was estimated to be 0.71 and 0.62 sec for the 3-P and 2-P nuclei, respectively.

percent DPG bound, a linear relationship was observed with both P nuclei (Fig. 1). The y-intercepts in Fig. 1 are similar to values observed in the absence of hemoglobin, indicating that no other factor adds significantly to the $1/T_1$ changes associated with DPG binding in model solutions. Since ATP and Mg^{2+} are cellular constituents that can affect DPG binding to hemoglobin (7), T_1 measurements were also determined in their presence (Expt. 4). The DPG/hemoglobin concentration ratio in Expt. 4 was intermediate between the values in Expts. 2 and 3 as were the values in percent DPG bound. On the other hand, the T_1s were generally higher, suggesting that a small interaction occurred; however, a similar trend between $1/T_1$ and percent DPG bound was observed under these conditions as well.

Following x-ray crystallography, the molecular interaction between DPG and deoxyhemoglobin has been described as taking place in the central cavity and involving N-terminal amino groups as well as other positively charged groups (8). It would seem in theory that information on binding at each point of interaction between DPG and hemoglobin should be attainable by NMR spectroscopy. In the present study, the T_1s of the 2-P and 3-P nuclei appeared to be associated with DPG binding to hemoglobin since a direct relationship was obtained between the $1/T_1$s of either the 2-P or 3-P nucleus and percent DPG bound in model solutions. Additional T_1 variations were seen which were consistent with changes in percent DPG bound due to deoxygenation or to different DPG concentrations. In deoxyhemoglobin, the heme Fe^{2+} is paramagnetic (9) so that it could theoretically lower T_1s in deoxygenated model solutions. However, this paramagnetic influence was not great enough to affect the direct relationship between $1/T_1$s and percent DPG bound since the points for deoxyhemoglobin lay on the same line as those for oxyhemoglobin. This suggests that the electron densities between the central cavity and the heme Fe^{2+} insulate the influence of the latter on the DPG present in the central cavity.

In intact erythrocytes, T_1 values were slightly greater than in model

solutions (Expt. 5-7 in Table 1) under similar conditions of pH, temperature, and DPG concentration so that the percent DPG bound may be lower within the red blood cells. If the 2-P line in Fig. 1 is used as a standard curve, the percent DPG bound in untreated and deoxygenated erythrocytes may be approximated as 12 and 23%, respectively. The value for deoxygenated cells is roughly similar to the value (40%) obtained from chemical shift measurements (2) but is markedly different from the value (90%) calculated from equilibrium constants of components associated with DPG binding (10). The basis for this discrepancy is as yet unclear.

The T_1s of the 3-P nucleus were lower than the 2-P T_1s in cell samples that were not deoxygenated. This result seems to suggest that the 3-phosphate may be bound more tightly than the 2-phosphate in these cells compared to model solutions or deoxygenated cells. In the absence of additional information, however, alternate explanations may be equally feasible. For example, the 3-P nucleus might be exposed to a paramagnetic oxygen nucleus in cellular systems due to site specific interactions at the higher hemoglobin concentration present inside the cell. In contrast to the cells that were not deoxygenated, the 3-P T_1 of deoxygenated cells was greater than the 2-P T_1 as it was in model solutions. Thus, while the 2-P T_1 decreased with deoxygenation as it did in model solutions, the 3-P T_1 increased as O_2 was removed from intact erythrocytes. This atypical T_1 variation of the 3-P nucleus indicates that the latter should not be used in estimating DPG binding in intact red cells.

The results of this study suggest that it is possible to determine DPG binding in intact cells for the deoxygenated and, most probably, for the oxygenated case by using the T_1 of the 2-P nucleus. The anomalous T_1 of the 3-P nucleus indicates that there are certain binding characteristics in cells that cannot be anticipated from studies of simple model solutions. The present investigation reinforces the view therefore that NMR spectroscopy

provides an important tool for the study of organic phosphate binding to hemoglobin in intact erythrocytes under various physiological and pathological conditions.

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REFERENCES

1. Costello, A.J.R., Marshall, W.E., Omachi, A. and Henderson, T.O. (1976) Biochim. Biophys. Acta 427, 481-491.
2. Marshall, W.E., Costello, A.J.R., Henderson, T.O. and Omachi, A. (1977) Biochim. Biophys. Acta 490, 290-300.
3. Drabkin, D.L. (1946) J. Biol. Chem. 164, 703-723.
4. Hurn, B.A.L. (1968) Storage of Blood, p. 137, Academic, London.
5. Canet, D., Levy, G.C. and Peat, I.R., (1975) J. Mag. Res. 18, 199-204.
6. Gupta, R.K., Benovic, J.L. and Rose, Z.B. (1979) J. Biol. Chem. 254, 8250-8255.
7. Gerber, G., Berger, H., Janig, G.R. and Rapoport, S.M., (1973) Eur. J. Biochem. 38, 563-571.
8. Perutz, M.F. (1970) Nature 228, 726-739.
9. Pauling, L. and Coryell, C.D. (1936) Proc. Natl. Acad. Sci. U.S.A. 22, 159-163.
10. Hamasaki, N. and Rose, Z.B. (1974) J. Biol. Chem. 249, 7896-7901.
11. Moon, R.B., Richards, J.H. (1973) J. Biol. Chem. 248, 7276-7278.