THE RATE OF DEOXYGENATION OF RED BLOOD CELLS: EFFECT OF INTRACELLULAR 2,3-DIPHOSPHOGLYCERATE AND PH*

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Received 30 June 1971

1. Introduction

The rate of deoxygenation of red blood cells has interested many investigators [2–15] since Roughton opened the field in 1932 [1]. It has been shown that the same factors which affect the dissociation of oxygen from hemoglobin in solutions, such as temperature, pH and carbon dioxide, exert similar effects in intact red blood cells [8, 9]. The recent elucidation of the influence of organic phosphates on the equilibrium between oxygen and hemoglobin, both in solution [16, 17] and in red blood cells [18], as well as their effects on the kinetics of oxygen association and dissociation of hemoglobin solutions [19, 20], led us to explore the effect of 2,3-diphosphoglycerate (2,3-DPG) on the rate of deoxygenation of intact human red blood cells. As a highly charged anion at physiologic pH, 2,3-DPG affects the distribution of protons across the cell membrane as predicted by Gibbs-Donnan theory [21]. Therefore, alterations in the content of 2,3-DPG will significantly modify intracellular pH. In order to isolate the effects of 2,3-DPG per se on deoxygenation from those mediated by protons, it was essential to redefine the effects of pH change alone on the rate of cellular deoxygenation. Our results suggest that 2,3-DPG does directly increase the deoxygenation rate of whole red blood cells in a manner which is independent of its effect on intracellular pH. However, as 2,3-DPG is increased above an equimolar concentration with hemoglobin, the observed increase in deoxygenation rate is attributed entirely to the lowering of intracellular pH by 2,3-DPG. Furthermore, we show that intracellular 2,3-DPG increases the "kinetic Bohr effect" of red cells thus further enhancing the effect of protons on cellular deoxygenation.

* Portions of this work were presented in abstract form elsewhere [29].

2. Materials and methods

Human venous blood was drawn in heparin, centrifuged, and the plasma removed and saved. The cells were washed and resuspended to a hematocrit of 10% in simple bicarbonate buffer. Incubations were performed at pH 7.2, 37°, with 20 mM phosphate, 20 mM pyruvate, and varying concentrations of inosine so as to achieve a wide range of intracellular 2,3-DPG

concentrations. After 16 to 24 hr, the cells were washed and the plasma was added back to the red cells to give a normal hematocrit. Intracellular 2,3-DPG concentrations were measured by the method of Keitt [22]. The pH gradient between the red cell and plasma, ΔpH, was measured by the method of Hilpert et al. [23], over the entire range of different 2,3-DPG concentrations. Because ΔpH is lower in Tris-HCl cell suspensions than in bicarbonate buffers or plasma [24], intracellular pH was measured directly on each sample used for kinetic studies at the various extracellular pH values. (For these latter measurements, the method of Hilpert et al. [23] was modified in that CO_2 equilibration was not performed, since extracellular pH was maintained by the Tris-HCl.)

The samples of reconstituted blood with various concentrations of 2,3-DPG were diluted in 10 volumes of Tris-HCl buffer immediately before the mixing experiments. The osmolarity of each buffer at various pH values was adjusted to exactly 290 m Osm as measured on an osmometer (Advanced Instruments). In one series of experiments, deoxygenation rates of samples with varying concentrations of intracellular 2,3-DPG were measured at a constant extracellular pH of 7.5. A second series of experiments employed three sets of cells, one at high intracellular 2,3-DPG (61 μmole/g Hb), one at normal 2,3-DPG (13 μmole/g Hb), and one at very low 2,3-DPG (2 μmole/g Hb which was produced by incubation for 16 hr without substrate). Deoxygenation rates were measured on each sample at various extracellular pH values range from 6.6 to 8.5. The deoxygenation rate was measured spectrophotometrically at 22° in a modified Durrum-Gibson stopped flow apparatus using sodium dithionite as a deoxygenating agent. The modifications of the original Gibson apparatus [25] included the use of (1) a head-on photomultiplier tube, (2) a 2 mm cuvette with a straight path from the mixing jet to the stop syringe, and (3) different valves to minimize hemolysis.

Previous studies of whole cell suspensions [4–9] have utilized split beam techniques to cancel out possible changes in turbidity resulting from turbulence or a change in cell volume deoxygenation. The effectiveness of the short path length and head-on photomultiplier tube in detecting both transmitted and scattered light was determined in two ways. First, the deoxygenation reaction was followed at an isosbestic point for hemoglobin (585 nm) and no significant change in transmittance could be detected. Secondly, the effect of gross shrinking and swelling of the cells on transmittance was determined at the normal reaction wavelength (577 nm). This was accomplished by mixing isotonic cell suspensions with equal volumes of 0.5, 1.0, and 2.0 times isotonic Tris-HCl in the absence of dithionite. The change in transmittance due to these gross changes in shape were equal to or less than the total change in transmittance noted with dithionite in isotonic buffer. It was concluded that the minor changes in cell volume occurring on deoxygenation, contributed a negligible amount to the observed transmittance changes during deoxygenation. The total change in transmittance due to deoxygenation was calibrated using the totally saturated and desaturated cells (by adding crystals of dithionite) at the same hematocrit to determine that the observed reaction began at near 100% saturation and reached total desaturation at the end of its course. This was found to be the case. For the studies reported herein, from three to five mixing experiments were performed per experimental point reported. The data was analyzed by determining a rate constant, k_c in sec^{-1}, assuming a first order deoxygenation process. This assumption was found to be valid over the saturation range analyzed (100 to 30%).

3. Results

Fig. 1 illustrates a plot of the measured ΔpH versus the concentration of intracellular 2,3-DPG. The line is a linear least squares fit of the data and the slope and intercept at zero 2,3-DPG agree closely with that of Duhm in similar studies [26]. It can be observed that an increase in intracellular 2,3-DPG induces a nearly linear increase in ΔpH over the entire range of 2,3-DPG concentrations studied. In addition, we have observed that when the same measurements are made in isotonic Tris-HCl, in the absence of CO_2, the values for ΔpH at a given 2,3-DPG level are significantly lower than in bicarbonate, in agreement with the results of Battaglia et al. [24]. Fig. 2 is a plot of intracellular 2,3-DPG concentration versus k_c. It can be seen that as the intracellular 2,3-DPG concentration is elevated, the value of k_c increases in an apparent hyperbolic fashion. However, as shown in fig. 3, when the values are corrected for intracellular pH, no dif-

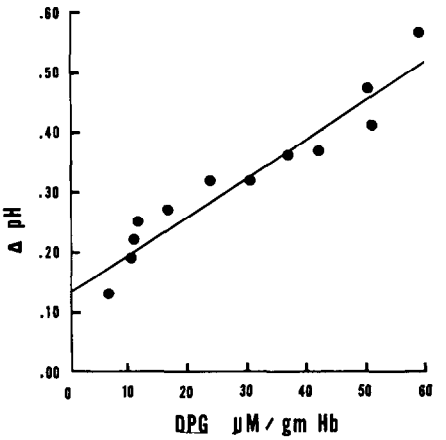

Fig. 1. Plot of ΔpH versus intracellular concentration of 2,3-DPG as measured by the method of Hilpert et al. [23] in bicarbonate buffer. Linear least squares fit of the data gave the equation ΔpH = 0.007 (DPG μmoles/g Hb) + 0.129.

ference in deoxygenation rate is observable between cells with normal (13 μmole/g Hb) and 5 times normal (61 μmole/g Hb) levels of 2,3-DPG. In marked contrast, both normal and high 2,3-DPG cells are much faster than low 2,3-DPG cells (2.0 μmole/g Hb) below about pH 7.7. These results suggest that a direct effect of 2,3-DPG is present only up to about an equimolar concentration with hemoglobin. It is further

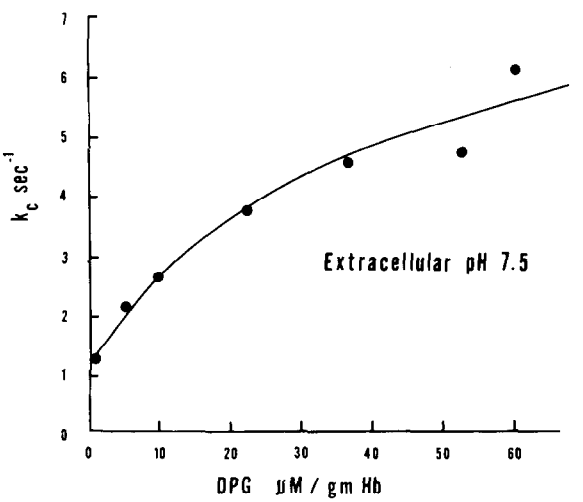

Fig. 2. Plot of the deoxygenation rate constant (k_c in sec^{-1}) versus the concentration of intracellular 2,3-DPG at constant extracellular pH 7.5 and 22° in isotonic Tris-HCl.

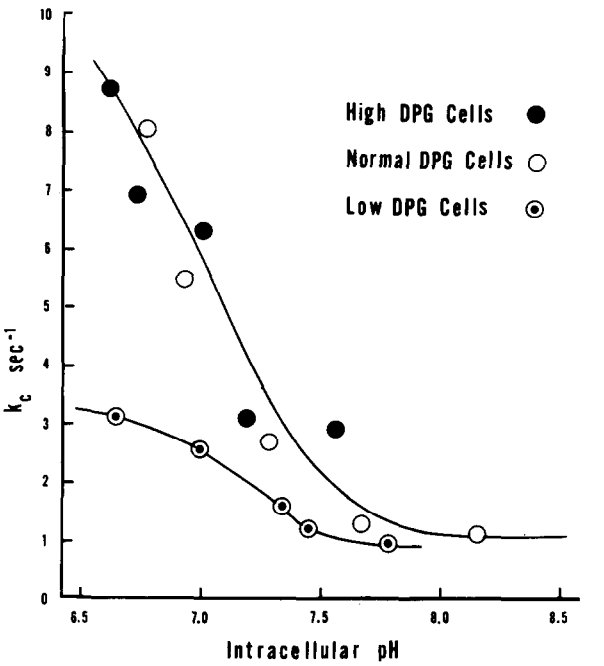

Fig. 3. Plot of k_c in sec^{-1} versus measured intracellular pH in isotonic Tris-HCl at various extracellular pH values and 22°. Concentration of 2,3-DPG: • = 61 μmole/g Hb; ○ = 13 μmole/g Hb; ⊙ = 2.0 μmole/g Hb.

noted, using the data of fig. 3, that the "kinetic Bohr effect" (defined as Δ log k_c/ΔpH) is also increased up to equimolar concentrations of 2,3-DPG over the pH range 6.6 to 7.2 (−0.667 as compared to −0.404 respectively). The values of k_c reported herein are nearly comparable to those reported by Lawson [9]. However, direct comparison cannot be made since his values are reported versus extracellular pH, in the presence of CO_2 and, of course, the levels of 2,3-DPG were not measured.

4. Discussion

Sirs [7] has reported variations in the overall rate of deoxygenation, k_c, of red blood cells stored in acid citrate-dextrose solution compared to normal fresh blood. Among other findings, he noted that the overall deoxygenation rate was significantly lower in the stored cells as compared to fresh cells, and that incubation in adenosine restored normal function. It is well

known that acid citrate-dextrose blood becomes rapidly depleted in 2,3-DPG content and that its level can be partially restored by adenosine incubations. Thus the observations of Sirs [7] may be explained by differences in 2,3-DPG within the stored and fresh red blood cell. In the same paper, Sirs [7] noted that the initial rate constant for deoxygenation, k_4, in the reaction $Hb_4O_8 \xrightarrow{k_4} Hb_4O_6 + O_2$, as measured by carbon monoxide replacement techniques, did not vary with storage of the cells. Because k_4, obtained in this way, is a direct measurement of the chemical dissociation of the first oxygen molecule from oxyhemoglobin, while k_c is composed of many additional variables [3, 8, 9], Sirs [7] concluded that factors other than a change in the chemical reactions of hemoglobin and oxygen were responsible for the change in k_c with storage.

More recent evidence [27, 11] has shown that k_4 is unaffected by alterations in 2,3-DPG or protons. Since alterations in 2,3-DPG and attendant changes in intracellular pH are probably responsible for the differing functional properties of stored and fresh cells, Sirs' observation of an invariant k_4 does not rule out altered chemical reactivity of hemoglobin as an explanation for a decreased k_c. Indeed, all data collected to date, including the present studies, remain compatible with a direct effect of 2,3-DPG and protons on chemical reactivity of hemoglobin in intact cells, but only after dissociation of the first oxygen molecule. This discrepancy between k_4 and k_c seems to suggest that oxygenation and deoxygenation within the red cell cannot be treated as a one-step equilibrium (i.e. $HbO_2 \rightleftharpoons Hb + O_2$).

As noted in fig. 3, at a constant intracellular pH, 2,3-DPG increases k_c only up to approximately a one to one molar ratio of 2,3-DPG to hemoglobin. This is in striking contrast to previous studies of deoxygenation of hemoglobin solutions [19, 20], where 2,3-DPG continues to increase the rate at concentrations far above equimolar. However, as implied by Nicolson and Roughton [2], the intact cell and hemoglobin solutions are not strictly comparable systems with respect to deoxygenation rates measured with dithionite. For hemoglobin solutions, the rate of fall of oxygen tension with dithionite, is much faster than the rate of deoxygenation such that oxygen binding constants do not contribute to the overall rate. In red cell suspensions, on the other hand, intracellular oxygen tension falls more slowly, allowing rebinding of this oxygen to intracellular hemoglobin during the cellular deoxygenation process. Therefore, both intracellular hemoglobin association and dissociation constants contribute to the overall rate of deoxygenation as demonstrated by Lawson [9].

The observation that 2,3-DPG alters the "kinetic Bohr effect" is in agreement with the equilibrium studies of Tomita and Riggs [28] who also showed that 2,3-DPG increases the true Bohr effect of hemoglobin up to, but not above, equimolar concentrations. Thus it appears that the increase in k_c with occurs above equimolar concentrations of 2,3-DPG (fig. 2) is not a result of any direct interaction between 2,3-DPG and hemoglobin but reflects only the increase in quantity of intracellular protons consequent to the Donnan equilibrium. Other workers have pointed out this dual role of 2,3-DPG on whole blood-oxygen equilibrium curves [21, 26]. However, the relative roles of 2,3-DPG and protons have yet to be as clearly delineated as in the present studies.

Acknowledgements

This work was supported by grants from the Florida Heart Association and the Veterans Administration, as well as contract number N00014-68-A-0173-004 between the Office of Naval Research and the University of Florida. We thank Mr. J.E. Martinez for aid in processing the data through the computer. One of us (ASK) was supported by a teaching and research scholarship from the American College of Physicians.

References

[2] F.J.W. Roughton, Proc. Roy. Soc. (London) B111 (1932) 1.
[2] P. Nicolson and F.J.W. Roughton, Proc. Roy. Soc. (London) B138 (1951) 241.
[3] F.J.W. Roughton, Progr. Biophys. Biophys. Chem. 9 (1959) 55.
[4] J.A. Sirs, Biochim. Biophys. Acta 112 (1966) 538.
[5] J.A. Sirs, Biochim. Biophys. Acta 126 (1966) 19.
[6] J.A. Sirs, Biochim. Biophys. Acta 126 (1966) 28.
[7] J.A. Sirs, J. Physiol. 203 (1969) 93.
[8] W.H. Lawson, R.A.B. Holland and R.E. Forster, J. Appl. Physiol. 20 (1965) 912.
[9] W.H. Lawson, J. Appl. Physiol. 21 (1966) 905.

[10] W. Niesel, G. Thews and D. Lubers, Pflügers Arch. Physiol. 268 (1959) 296.
[11] Q.H. Gibson, F. Kruezer, E. Meda and F.J.W. Roughton, J. Physiol. (London) 129 (1955) 65.
[12] R.C. Menke and D. Hershey, in: Blood Oxygenation ed. D. Hershey (Plenum Press, New York, 1970) p. 72.
[13] J.A. Sirs, ibid. p. 116.
[14] J.W. Legge and F.J.W. Roughton, Biochem. J. 47 (1950) 43.
[15] T. Nakamura and N.C. Staub, J. Physiol. (London) 173 (1964) 161.
[16] A. Chanutin and R.R. Curnish, Arch. Biochem. Biophys. 121 (1967) 96.
[17] R. Benesch and R.E. Benesch, Biochem. Biophys. Res. Commun. 26 (1967) 162.
[18] G. Duc and K. Engel, Scand. J. Clin. Lab. Invest. 24 (1969) 405.
[19] J.M. Salhany, R.S. Eliot and H. Mizukami, Biochem. Biophys. Res. Commun. 39 (1970) 1052.
[20] Q.H. Gibson, Biochem. Biophys. Res. Commun. 40 (1970) 1319.
[21] F.C. Battaglia, H. McGaughey, E.L. Makowski and G. Meschia, Amer. J. Physiol. 219 (1970) 217.
[22] A.S. Keitt, J. Lab. Clin. Med. 77 (1971) 470.
[23] P. Hilpert, R.G. Fleischmann, D. Kempe and H. Bartels, Am. J. Physiol. 205 (1963) 337.
[24] F. Battaglia, E.L. Makowski, G. Meschia and M.M. Niernberg, Pediat. Res. 2 (1968) 193.
[25] Q.H. Gibson, Discussions Faraday Soc. 17 (1954) 137.
[26] J. Duhm, Pflügers Arch. Physiol. 319 (1970) 2.
[27] Q.H. Gibson and R.D. Gray, Biochem. Biophys. Res. Commun. 41 (1970) 415.
[28] S. Tomita and A. Riggs, J. Biol. Chem. 246 (1970) 547.
[29] A.S. Keitt, J.M. Salhany and R.S. Eliot, Ann. Intern. Med. 74 (1971) 827.