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The Separate Effects of H^+ and 2,3-DPG on the Oxygen Equilibrium Curve of Human Blood

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SUMMARY. Addition of non-saturating amounts of 2,3-DPG (2,3-diphosphoglycerate) within the red cell (2,3-DPG/haemoglobin less than 1) initially reduces Hill's parameter, *n*. With increasing 2,3-DPG/haemoglobin, *n* increases until a maximum is reached at 2,3-DPG/haemoglobin greater than 1. Thus, 2,3-DPG influences the shape as well as the position of the whole blood oxygen equilibrium curve (OEC). The importance of this effect on the oxygen carrying capacity of the blood is considered. The effect of 2,3-DPG on the position of the OEC (p50, the pO_2 at one-half maximal O_2 saturation) is via its allosteric effect on haemoglobin at 2,3-DPG/haemoglobin less than 1. Above that ratio, its effect is to reduce intracellular relative to the extracellular pH.

Investigations into the functional properties of haemoglobin (Hb) have been carried out by two broad groups of investigators: those interested in the physical-chemical properties of Hb and those interested in the functional properties of blood. In general, the former have studied purified solutions under controlled conditions, and the latter have sought overall effects so that the capability of the blood to deliver oxygen to tissues could be studied. Many factors within the red cell influence the functional properties of Hb; some are well known and some are poorly understood. For example, in addition to the influence of 2,3-DPG (2,3-diphosphoglycerate) on oxygen affinity due to its allosteric effect, it can alter red cell pH (pH_i) by its effect on the Donnan equilibrium of the membrane (Duhm, 1972).

Recent studies have considered the behaviour of the blood p50 (the oxygen pressure at half maximal oxygen saturation) obtained by measuring pO_2 and saturation for a few points around p50 and using the equation of Hill:

$$\log(Y/1-Y)=n\log P+K \qquad (1)$$

where Y is the fractional saturation of Hb with oxygen and P is pO_2. An assumed value of n is usually used to extrapolate the curve into the physiologically important range.

We have recently described a new method for the measurement of the whole blood OEC

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which is automatic, rapid and measures the continuous curve over the entire range 0–150 mmHg pO_2 at fixed plasma pH (pH_e) and pCO_2 (Winslow, 1975). This method has the advantage that no assumptions about the shape of the curve (i.e. fixed n) need be made.

Because of the difference in pH_i and pH_e, both are needed as a function of 2,3-DPG concentration to study either the biochemical properties of Hb within the cell or the physiological properties of blood. In the present paper we present a nomogram relating pH_e, pH_i and 2,3-DPG concentration of human blood and a series of measurements of p50 and n values obtained under various conditions of pH_e and 2,3-DPG concentration.

MATERIALS AND METHODS

Haematologic Measurements

Heparinized (50 units/ml) blood from two non-smoking, adult male donors (MS and RW) was used for all the experiments. Measurement of total Hb concentration, carboxyhaemoglobin (COHb) and Methaemoglobin (MetHb) were performed using the Microblood Analyzer (Carlo Erba Strumentazione, Milan, Italy; for further details see Rossi-Bernardi *et al*, 1977). These values fell within the normal range. 2,3-DPG concentration was measured according to Nygaard & Rørth (1969), using kits supplied from Calbiochem. The reproducibility of this method in our laboratory is ±10% (see Winslow *et al*, 1978).

Alteration of 2,3-DPG Concentration

For experiments with unaltered 2,3-DPG concentration, whole blood was used within 1 h after venipuncture. High 2,3-DPG levels were obtained using the method described by Deuticke *et al* (1971). According to this method it was possible to reach a 2,3-DPG/Hb molar ratio of 2.8 after a 90 min sterile incubation at 37°C with an IPP (inosine: pyruvate: phosphate in various ratios) solution isotonic with the cells. No haemolysis occurred and COHb and MetHb concentrations were not significantly different from the values found *in vivo*.

2,3-DPG depletion was accomplished by sterile incubation of the cells at 37°C in isotonic saline over a period varying from 3 to 14 h depending upon the degree of depletion required. Air, humidified at 37°C, was passed into the incubation flask, and after incubation no change in Hb, MetHb, or COHb could be detected. Cells were resuspended in their native plasma before the experiment.

Intracellular pH Measurements

Small amounts of blood, usually 1 ml, were equilibrated for 15 min with selected gas mixtures at 37°C in an IL-237 tonometer (Instrumentation Laboratories, Lexington, Mass.). Gas mixtures were obtained from Lif-O-Gen Inc. (Cambridge, Md.) and their compositions were certified nominally to ±0.02%. After the equilibration time, the extracellular or plasma pH (pH_e) was measured directly using a microelectrode (IL 213, Instrumentation Laboratories, Lexington, Mass.). The remaining portion of the sample was anaerobically transferred with a Hamilton gas tight syringe (Hamilton Company, Reno, Nev.) into two small microcentrifuge tubes and sealed with a drop of paraffin oil. The tubes were then centrifuged for 3 min to sediment the red cells. The cells were frozen and thawed twice in dry ice and cold water respectively to assure total haemolysis and the pH of the erythrolysate was finally determined.

In order to achieve a wide range of pH_e (6.8–7.8), plasma pH was altered by additions of various amounts of isotonic HCl or $NaHCO_3$ directly into the tonometer. The additions were performed 5 min after the tonometry began, so that the system was maximally buffered to avoid haemolysis and MetHb formation due to abrupt changes in pH. All pH measurements were carried out in duplicate and occasionally quadruplicate and the results were expressed as ΔpH ($pH_e - pH_i$) versus pH_e. Six sets of experimental points at different 2,3-DPG ratios (0.2, 0.7, 1.0, 1.3, 1.8, 2.8) were studied and at least six measurements were carried out at each ratio. The 2,3-DPG ratio refers to the molar ratio of 2,3-DPG to Hb.

Oxygen Equilibrium Curves

Complete oxygen equilibrium curves were measured according to the previously described automated method (Winslow *et al*, 1978). Five different 2,3-DPG/Hb ratios were used (0.04, 0.4, 1.0, 2.0 and 2.5). At least six curves were run at each 2,3-DPG/Hb ratio at various pH_e's. The pH_e of the sample was varied before the run as follows: 2.5 ml of blood were centrifuged for 5 min at 2000 rpm in a refrigerated centrifuge. The supernatant plasma was introduced into the tonometer and small amounts of 4.4 molar lactic acid or 10 molar NaOH were added to it. After a 5 min equilibration with 5.6% CO_2 (balance N_2), packed red cells were added back to the plasma in the tonometer and the remainder of the procedure previously described was followed.

The p50 was obtained as the pO_2 at which 1/2 saturation occurred, and the slope of the Hill plot, n, was calculated at p50 by fitting data points between 45 and 55% saturation to equation 1 (approximately 30 points).

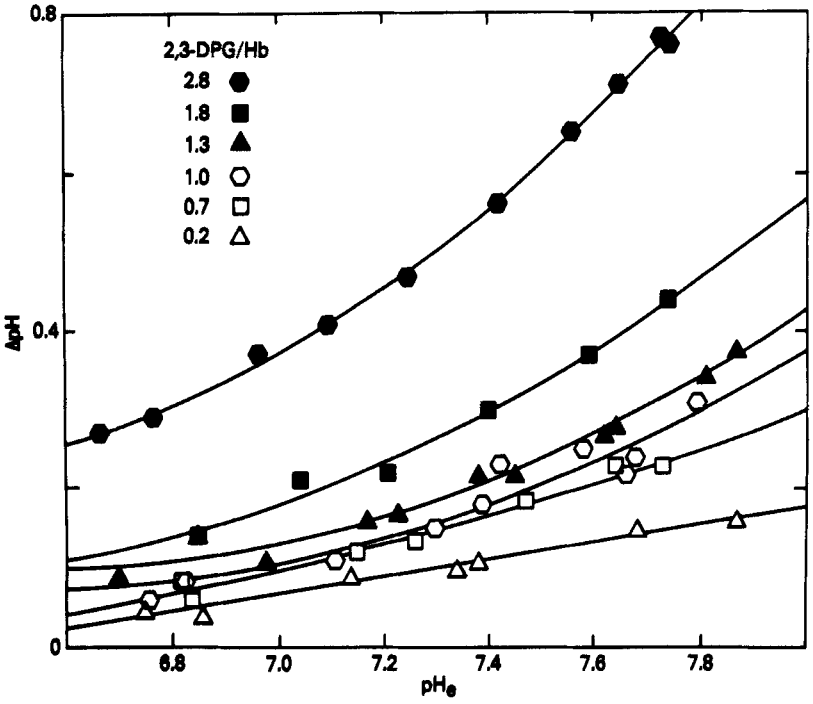

FIG 1. ΔpH ($pH_e - pH_i$) vs pH_e in human blood at different 2,3-DPG/Hb ratios.

RESULTS

Intracellular pH

pH_i is lower than pH_e under all conditions studied (Fig 1). When 2,3-DPG concentration increases, the difference between pH_e and pH_i (ΔpH) increases. In agreement with others (Gattinoni & Iapichino, 1974) we have found that CO_2 has essentially no effect on ΔpH (unpublished data). Takano *et al* (1976) found that pH_i of fully oxygenated blood was 0.035 units lower than that of fully deoxygenated blood. To eliminate this variable, all of the data in Fig 1 were obtained on fully oxygenated blood. All of the data points in Fig 1 (150 determinations for 50 conditions of 2,3-DPG and pH_e) were used to prepare a nomogram (Fig 2) which relates pH_e, pH_i and 2,3-DPG concentration. ΔpH (and hence pH_i) can be estimated with at most ± 0.015 pH units (SD) error. This nomogram is useful in studying the physical-chemical properties of Hb within the red cell, as described below.

Blood Oxygen Equilibrium Curves

As discussed previously (Winslow *et al*, 1978), some variation in p50 is to be found among

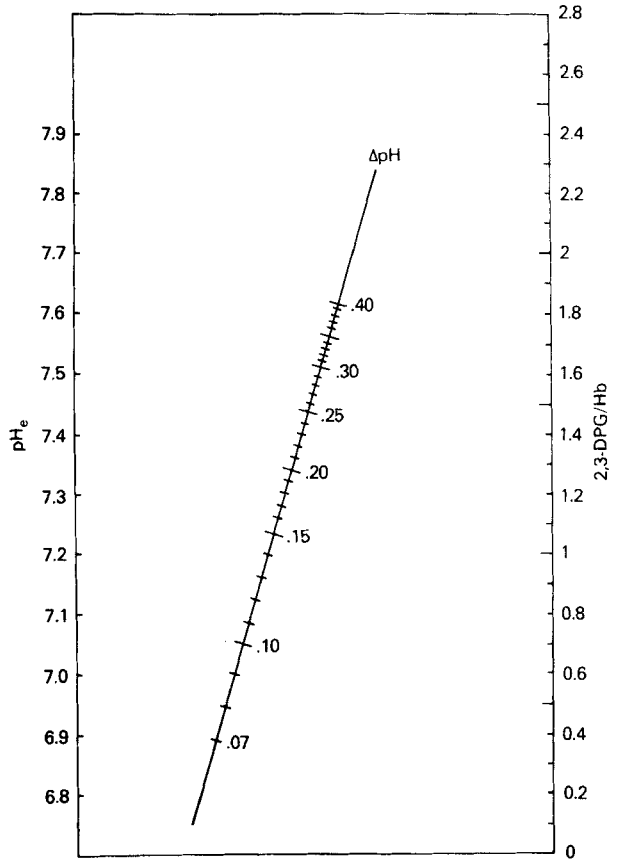

FIG 2. The pH_e, pH_i, 2,3-DPG nomogram. A straight line from measured value of pH_e and 2,3-DPG concentration passes through ΔpH.

normal individuals and therefore all of the present data have been obtained on blood from two subjects. These samples gave virtually identical curves under comparable conditions.

The Effects of H^+ on the OEC

In Fig 3A log p50 is plotted against pH_e to demonstrate the alkaline Bohr effect at fixed pCO_2 of 39 mmHg and various 2,3-DPG concentrations. This type of analysis gives information of physiological value because it employs *in vivo* conditions to study the behaviour of Hb with respect to pH_i. The nomogram in Fig 2 was used to calculate pH_i for each point. The experimental points of Fig 3B were fitted to straight lines using linear regression analysis to obtain Bohr factors (Δ log p50/ΔpH) at different concentrations of 2,3-DPG. We find that

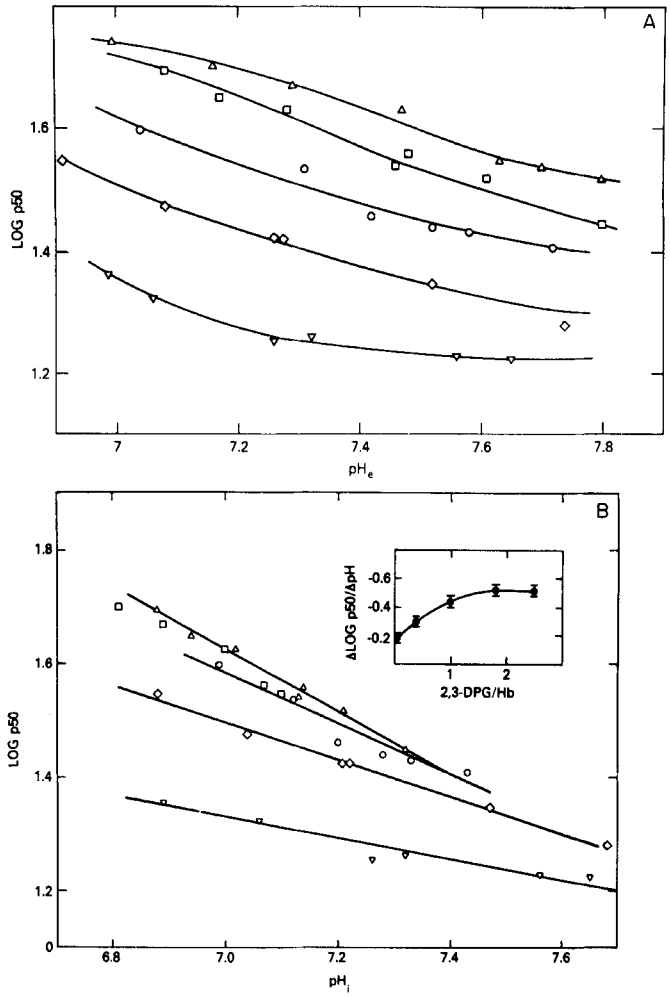

FIG 3. The alkaline Bohr effect. Log p50 is plotted against pH_e (A) or, after use of pH_e, 2,3-DPG concentration, and the nomogram of Fig 2, against pH_i (B). 2,3-DPG/Hb ratios are 0.04 (▽), 0.4 (◇), 1 (○), 1.8 (□) and 2.5 (△). Inset: The Bohr effect at varying 2,3-DPG/Hb ratios. Linear regression analysis of the data in Fig 3B was used to obtain Bohr factors (Δlog p50/ΔpH) at each 2,3-DPG concentration, pH_i 7.0. The error bars refer to the standard errors from the regression analysis.

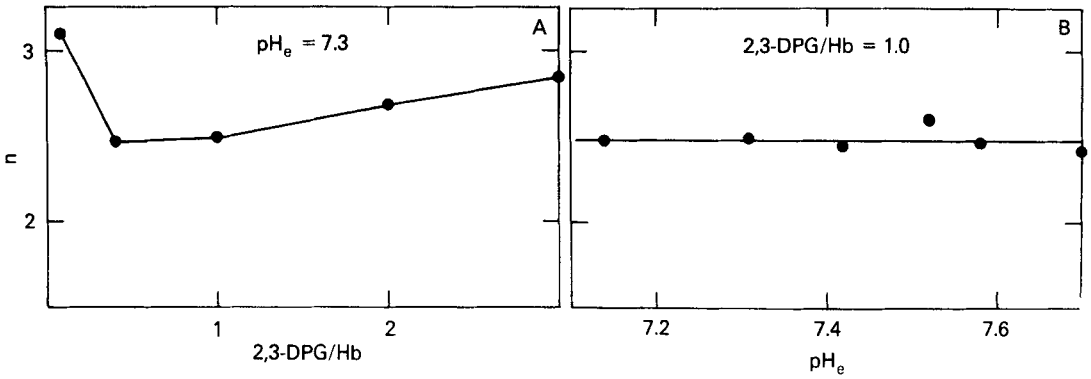

FIG 4. The effect of pH_e and 2,3-DPG on the shape of the OEC. Hill's parameters (n) were obtained as the slope of the Hill plot (log pO_2 vs log $(Y/1-Y)$ at 50% saturation. n is shown as a function of 2,3-DPG/Hb ratio at fixed pH_e (A) or as a function of pH_e, at fixed 2,3-DPG/Hb ratio (B).

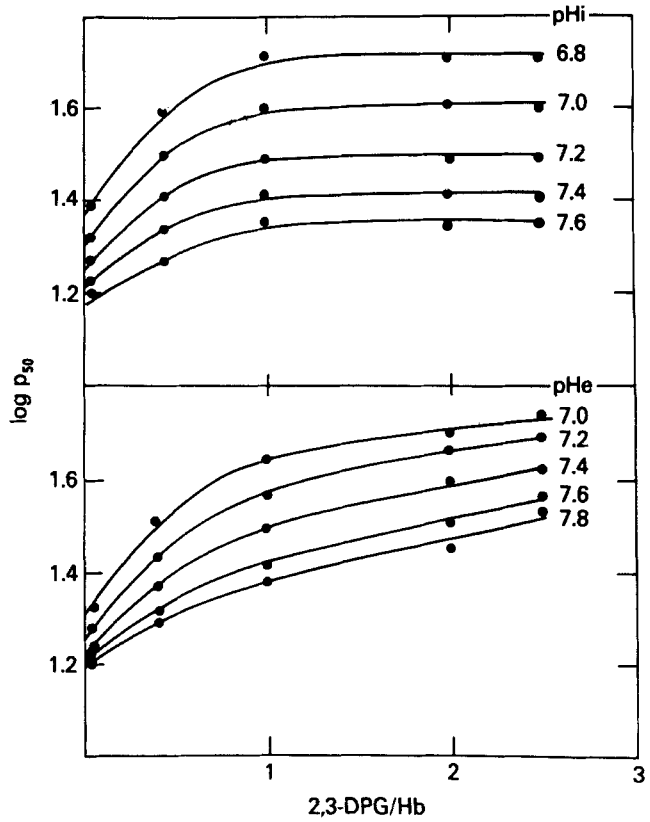

FIG 5. Log p50 vs. 2,3-DPG/Hb at fixed pH_i and pH_e.. The saturation effect of 2,3-DPG on the p50 is seen in the top panel at 2,3-DPG/Hb greater than 1.

increasing 2,3-DPG/Hb concentration in whole blood increases the Bohr factor up to a 2,3-DPG/Hb ratio of about 1.5, but beyond that ratio does not change (Fig 3B, inset). The increased Bohr effect in the presence of 2,3-DPG has been noted by others (Benesch *et al*, 1969; Duhm & Gerlach, 1973) and can be explained by the binding of 2,3-DPG to oxyhaemoglobin (Arnone, 1972).

H^+ appears to have very little effect on the slope of the OEC, as represented by Hill's parameter, n (Fig 4B), in the pH range 7.2–7.7. This is entirely consistent with the earlier result of Bunn & Guidotti (1972) with Hb solutions.

The Effect of 2,3-DPG on the OEC

The effect of 2,3-DPG on the shape of the OEC can be seen in Fig 4A. At fixed pH_e the effect of 2,3-DPG is to reduce Hill's parameter, which increases when 2,3-DPG concentration increases. Alteration of subunit cooperativity may be more apparent than real, since reduced n can occur when two species are present: in this case Hb molecules which either bind 2,3-DPG or which do not. Such biphasic OEC's would be expected at 2,3-DPG/Hb ratios less than 1 (Imai & Tyuma, 1973). The effect of 2,3-DPG on the position of the OEC can be estimated from its effect on the p50 (Fig 5). When plotted at pH_i, p50 increases with 2,3-DPG concentration up to 2,3-DPG/Hb=1, and then has no further effect, even at 2,3-DPG/Hb=2.5. This is in agreement with experiments using Hb solutions (Benesch *et al*, 1969). In contrast, when plotted vs. pH_e, p50 continues to increase at 2,3-DPG/Hb greater than 1. This can be explained by the data of Fig 1, in which 2,3-DPG raises p50 by two different mechanisms: its direct allosteric effect on Hb and by increasing ΔpH.

DISCUSSION

It is well known that in some pathological and adaptive conditions 2,3-DPG concentration within the red cell can vary widely. Some of these are anaemia, hexokinase and pyruvate kinase deficiencies, acidosis, shock, massive blood tranfusion, congenital and acquired heart disease, chronic lung disease, and high altitude adaptation. In addition, there is a close relationship between ΔpH and 2,3-DPG concentration. Therefore, to study the function of Hb under various circumstances, it is essential to know the relations between pH_e, pH_i and 2,3-DPG concentration.

The mechanism of regulation of ΔpH is not fully quantifiable at present, because all of the factors which make up the Donnan equilibrium under a given set of conditions are involved (Duhm, 1972). Thus, the data of Fig 1 are intended only to be empirical relations.

2,3-DPG has a strong effect on the position (p50) and shape (n) of the OEC. However, when ΔpH is fully considered, our results show that if 2,3-DPG/Hb is greater than 1, the effect of DPG on p50 is entirely by its effect on pH_i. This is in agreement with the X-ray studies of Arnone (1972), who demonstrated the stereochemical basis of binding of 2,3-DPG to deoxyhaemoglobin in a 1:1 molar ratio.

Many previous workers have observed the increase in the alkaline Bohr effect of haemoglobin due to 2,3-DPG (Benesch *et al*, 1969; Duhm, 1976; Tomita & Riggs, 1971). The mechanism of this increase is thought to be the increased protonation of deoxy Hb when 2,3-DPG is bound (Riggs, 1971). The binding of 2,3-DPG to Hb is strongly influenced by pH, ionic strength, and

the presence of competing anions (such as chloride) (Deal, 1973) and therefore the behaviour of haemoglobin solutions and whole blood in terms of the 2,3-DPG influence on the Bohr effect might not be expected to be quantitatively equivalent. We find a maximum Bohr factor (-0.50) for whole blood (relative to pH_i) above DPG/Hb=1, while the maximum for haemolysate was found by Benesch *et al* (1969) at a much lower 2,3-DPG/Hb ratio.

These findings suggest that the general behaviour of the 2,3-DPG-Hb system within erythrocytes is qualitatively described by the model of Riggs (1971) but quantitatively much more complex. For an exact description, the individual effects of high Hb concentration, CO_2, other small anions and allosteric effectors within the cell will have to be described in detail. Nevertheless, an important physiologic observation can be made: the maximum Bohr effect appears to occur under conditions which are found in normal blood, 2,3-DPG/Hb about 1.

Bunn & Guidotti (1972) and Tyuma *et al* (1971) found a dependence of Hill's coefficient, n, on the presence of organic phosphates in Hb solutions. Low 2,3-DPG concentrations (2,3-DPG/Hb less than 1), however, are associated with biphasic oxygenation curves (Imai & Tyuma, 1973). Thus, under such circumstances, 2 Hb species are present, Hb and HbDPG. Since Hb has high oxygen affinity, and HbDPG low, the former oxygenates first at low pO_2 giving the effect of lowering n. This phenomenon does not necessarily imply decreased subunit cooperativity in either Hb species and therefore the usual significance of n in terms of cooperativity does not apply to our data when 2,3-DPG/Hb is less than 1.

Non-saturating amounts of 2,3-DPG within the cell would mean that many mathematical models of Hb oxygenation will not apply, because they require that only one Hb species be present. Furthermore, if the oxygenation curve is truly biphasic, extrapolation by Hill's equation to the physiological range of pO_2 is not valid since n is not constant over the entire saturation range (Tyuma *et al*, 1972). Thus, large errors could result from calculating arterial–venous oxygen differences assuming constant n (Neville, 1977).

Greater precision in the quantitative description of the whole blood OEC must await further developments in the mathematical description of the OEC under different conditions of physiological importance.

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