

# Glycosylated Haemoglobins and the Oxygen Affinity of Whole Blood

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**Summary.** The  $pO_2$  at which haemoglobin is half-saturated with oxygen ( $p50$ ) was determined at fixed  $pCO_2$  (45 mmHg) and without altering the resulting pH and the level of organic phosphates in heparinized whole blood samples from 26 diabetic patients and 24 normal subjects of both sexes. Diabetic blood  $p50$  was higher ( $29.79 \pm 1.68$  versus  $28.26 \pm 1.16$  mmHg,  $p < 0.001$ ) and with a higher 2,3-diphosphoglyceric acid/haemoglobin molar ratio ( $1.04 \pm 0.15$  versus  $0.86 \pm 0.10$ ,  $p < 0.001$ ). The pH at a  $pCO_2$  of 45 mmHg was the same in the two groups. The observed  $p50$  values were compared

with those obtained after normalization in respect to pH and the level of 2,3-diphosphoglyceric acid. We conclude that glycosylated haemoglobins, known to have an increased affinity for oxygen when purified and in diluted solutions, do not play a significant role in the oxygen affinity pattern of diabetics at the concentrations normally found in vivo.

**Key words:** Glycosylated haemoglobins,  $HbA_{1c}$ , blood oxygen affinity, 2,3-diphosphoglyceric acid, blood gases.

Glycosylated haemoglobins ( $HbA_1$ ), of which the most abundant fraction is  $HbA_{1c}$ , are known to occur at higher levels in diabetic than in normal blood [1], and thus to be an index of diabetic control [2]. Purified and diluted solutions of  $HbA_{1c}$ , in presence of organic phosphates at pH 7.0 and 20 °C, show a higher affinity for oxygen in respect to normal haemoglobin [3]. Many authors [2, 4–6] have extrapolated these results, hypothesizing that in diabetic blood in vivo there is a higher oxygen affinity compared with normal blood under the same conditions. Direct experimental support for this hypothesis is, however, ambiguous, mainly because the other factors known to affect haemoglobin oxygen affinity (hydrogen ions, carbon dioxide, and organic phosphates) interfere with the postulated effect of  $HbA_{1c}$ , which is therefore not easily detectable [7, 8].

The purpose of this investigation was to evaluate the effect of glycosylated haemoglobins on the regulation of the oxygen affinity in whole blood from diabetic subjects. This has been made possible by a new method for determining the  $p50$  (the  $pO_2$  at which haemoglobin is half-saturated with oxygen) with an accuracy of  $\pm 0.3$  mmHg [9]. The equations reported here and in the literature [9] for calculating the normal human blood  $p50$  from known concentrations of hydrogen ions, carbon dioxide, and 2,3-diphosphoglyceric acid (2,3-DPG) allowed the normalization of the observed  $p50$  values.

## Patients and Methods

### Patients

Twenty-six diabetics (12 males and 14 females) on insulin treatment were the subjects of this investigation. The patients were admitted to hospital for assessment of their glycaemic control. Smokers, ketotic and acidotic patients, patients with abnormal haemoglobins, methaemoglobinemia, renal failure, other diseases besides diabetes mellitus, and patients with regular drug intake other than insulin were excluded from the present study to avoid possible interferences by unidentified factors during evaluation of the oxygen affinity state.

The control group was 24 normal, healthy, non-smoking volunteers (13 males and 11 females) with no family history of diabetes. Informed consent was obtained from all subjects before venepuncture.

### Methods

One ml of blood was withdrawn from the antecubital vein 3–5 h after insulin administration (in the case of diabetic patients). Blood was treated with lithium-heparin and immediately placed in an ice-bath. All samples were analyzed within 2 h of venepuncture.

An aliquot of the sample (0.4 ml) was tonometered in a closed flask with known  $pO_2$ , at a fixed  $pCO_2$  of 45 mmHg at 37 °C, without addition of acids or bases. The haemoglobin saturation for oxygen and the  $p50$  were measured as previously described [9]. The  $p50$  value was normalized with respect to pH,  $pCO_2$  and the [2,3-DPG]/[Hb] molar ratio using the following equations:

$$\log p50_{pH\ 7.0} = (-0.69117 \times 10^{-3} \times pCO_2 + 0.3365) \times G + (0.3598 \times 10^{-3} \times pCO_2 + 1.599) \quad (1)$$

**Table 1.** Oxygen affinity of blood from normal subjects and diabetic patients at 37 °C, pCO<sub>2</sub> = 45 mmHg, unaltered pH and level of 2,3-DPG

	Normal subjects (n = 24)	Diabetic patients (all) (n = 26)	<i>p</i> <sup>a</sup>	Diabetic patients (present age < 30 years) (n = 8)	Diabetic patients (present age > 30 years) (n = 18)
Age (years)	25.7 ± 3.5	48.1 ± 25.8	—	23.6 ± 7.5	57.7 ± 12.2
Duration of diabetes (years)	—	9.3 ± 8.2	—	1.8 ± 1.3	12.3 ± 8.2
HbA <sub>1c</sub> (%)	7.25 ± 1.12	11.75 ± 2.40	< 0.001	12.18 ± 2.64	11.58 ± 2.36
p50 (mmHg)	28.26 ± 1.16	29.79 ± 1.68	< 0.001	29.70 ± 1.50	29.85 ± 1.88
pH	7.375 ± 0.027	7.361 ± 0.026	NS	7.358 ± 0.018	7.365 ± 0.028
[2,3-DPG]/[Hb] (mol/mol)	0.86 ± 0.10	1.04 ± 0.15	< 0.001	1.03 ± 0.06	1.05 ± 0.16
Normalized p50 (mmHg)	28.87 ± 1.25	30.96 ± 1.70	< 0.001	31.21 ± 0.88	30.90 ± 1.96
Δp50 <sup>b</sup>	0.61 ± 0.80	1.17 ± 1.23	NS	1.18 ± 1.27	1.10 ± 1.30
Hill's factor <sup>c</sup>	2.68 ± 0.07	2.66 ± 0.08	NS	—	—
MCHC (g/dl) <sup>d</sup>	35.1 ± 2.8	35.2 ± 2.5	NS	—	—

Results are expressed as mean ± SD

<sup>a</sup> *p* refers to the comparison of normal subjects versus all diabetic patients. NS = not significant (*p* > 0.1)

<sup>b</sup> Expressed as (normalized p50) - (experimental p50)

<sup>c</sup> Calculated on eight normal and 12 diabetic samples

<sup>d</sup> Mean corpuscular haemoglobin concentration, calculated on eight normal and 21 diabetic samples

$$\log p50_{pH\ 7.6} = (-0.138 \times 10^{-2} \times pCO_2 + 0.3607) \times G + (0.9089 \times 10^{-3} \times pCO_2 + 1.360) \quad (2)$$

$$\log p50_{pH} = \frac{(\text{pH} - 7.0) \times (\log p50_{pH\ 7.6} - \log p50_{pH\ 7.0})}{0.6} + \log p50_{pH\ 7.0} \quad (3)$$

where  $G = \log_{10} [2,3\text{-DPG}]/[\text{Hb}]$ .

For the assay of HbA<sub>1c</sub>, the haemolysate was obtained by a five-fold dilution of the blood into the haemolyzing agent (0.33% polyoxyethylene ether), and the fast haemoglobin fractions were separated using kits from Bio-Rad Laboratories, Richmond, California [10]. The total haemoglobin concentration was determined by the standard method of Drabkin [11]. The concentration of 2,3-DPG was measured in the blood sample simultaneously with determination of p50, using kits from Boehringer Biochemia, Milan, Italy, and was expressed as the molar ratio of 2,3-DPG to haemoglobin tetramer. The mean corpuscular haemoglobin concentration was measured using an S-Plus Coulter counter (Coulter Electronics, Hialeah, FL, USA). The level of carbonmonoxyhaemoglobin and of methaemoglobin were determined by a microblood analyzer (Carlo Erba Strumentazione, Milan, Italy).

The Hill's factor, *n*, was determined in eight normal and 12 diabetic samples of both sexes [12]. The oxygen saturation was measured at three different oxygen tensions, one near the p50, and the other two a few mmHg above and below the p50. The factor was calculated as the slope of the regression line when the experimental data were plotted as proposed by Hill.

For the statistical analysis of the results, Student's *t*-test for paired and unpaired data, linear regression and correlation coefficients were applied as appropriate.

## Results

The results are summarized in Table 1. Few differences were found between the two age classes of diabetic patients. As expected, the level of HbA<sub>1c</sub> was higher in diabetic patients than in control subjects [1]. The p50, measured at unaltered pH and 2,3-DPG level, was higher in diabetics. Blood pH after the tonometry was the

same in the two groups. The level of 2,3-DPG and the normalized p50 values were higher in diabetics. The difference (Δp50) between the normalized p50 and the experimental p50, represents the functional difference between the blood sample under investigation and the reference normal blood, and was similar in both groups. The Hill's factor and the mean corpuscular haemoglobin concentration, which is a factor possibly affecting the oxygen affinity of blood [13], were the same in both groups. The levels of carbonmonoxyhaemoglobin and of methaemoglobin never exceeded 2% and 1% respectively.

A systematic difference was found between males and females. The [2,3-DPG]/[Hb] ratio was always higher in females (0.92 ± 0.10 versus 0.84 ± 0.07, in normal subjects, *p* < 0.01; and 1.07 ± 0.15 versus 1.00 ± 0.10, in diabetic patients, *p* < 0.10). The increase of 2,3-DPG level corresponds to an increase of their p50 and Δp50 was similar for females and males (0.61 ± 1.13 mmHg versus 0.67 ± 0.51 mmHg among normal subjects; and 0.97 ± 1.46 mmHg versus 1.34 ± 0.96 mmHg among diabetic patients).

Eight of the diabetic patients were newly diagnosed. Their [2,3-DPG]/[Hb] ratio was 1.00 ± 0.06, and their Δp50 1.09 ± 1.26 mmHg. The values of p50 and Δp50 were not correlated with body weight or the duration of diabetes.

The level of 2,3-DPG was significantly correlated with the level of HbA<sub>1c</sub> ([2,3-DPG]/[Hb] = 0.70 + 0.02 × HbA<sub>1c</sub>, *r* = 0.51, *p* < 0.001, *n* = 50). Consequently the p50 value was also correlated with the level of HbA<sub>1c</sub> (p50 = 26.42 + 0.277 × HbA<sub>1c</sub>, *r* = 0.48, *p* < 0.001, *n* = 50). In contrast, there was a very weak correlation of Δp50 with HbA<sub>1c</sub> (Δp50 = 0.04 + 0.088 × HbA<sub>1c</sub>, *r* = 0.25, *p* < 0.1, *n* = 50).

## Discussion

The p50 is a useful parameter in defining the position of the oxyhaemoglobin dissociation curve. Hydrogen ions, carbon dioxide, and 2,3-DPG are its principal effectors [9]. In diabetic patients, the value of blood pH and pCO<sub>2</sub> depend on the value of the base excess, following the acid-base laws, but there are conflicting data in the literature about the levels of 2,3-DPG [14]. Arturson et al. [7] and Ditzel et al. [8] reported that the concentration of 2,3-DPG is increased in diabetics, and that the expected increase of p50 is compensated by the presence of larger amounts of HbA<sub>1</sub> and of HbA<sub>1c</sub>. These investigations were complicated by the difficulty in normalizing p50 with respect to pH, pCO<sub>2</sub>, and the level of 2,3-DPG.

In the present study, the p50 was measured in whole blood by a previously described method, with control of the main regulating factors [9]. The equations (1–3) were used to normalize the experimental data to the desired pH, pCO<sub>2</sub>, and 2,3-DPG level. Therefore it was possible to state if an apparently abnormal p50 could be explained by abnormalities of the base excess, the [2,3-DPG]/[Hb] ratio, or whether other unidentified factors (such as variant haemoglobins) might play a role. As the second possibility, the difference between the normalized p50 and the experimental p50,  $\Delta p50$ , might represent an index of functional abnormality of the blood sample under investigation.

The interaction coefficients of HbA<sub>1</sub> with the considered allosteric factors are presently unknown. However, the Hill's factor, the determination of which is independent of any assumption, is the same in both groups, implying that the haemoglobin molecule is functionally equivalent in the two groups of subjects. We assumed therefore that the reported equations represent the situation in the blood of diabetic patients.

Table 1 shows that  $\Delta p50$  is similar in the two groups, suggesting that factors other than hydrogen ions, carbon dioxide, and 2,3-DPG, such as HbA<sub>1</sub>, do not play any significant role in the oxygen affinity regulation in diabetics. Patients with HbA<sub>1</sub> levels above 15–16% could not be included in this investigation because of the possibility that limitations in their diet and physical activity lead to an altered p50 per se. A few samples with HbA<sub>1</sub> > 16% (not included in Table 1 because of the exclusions stated) do however show almost complete overlapping with the data in Table 1. The subdivision of diabetics in two age classes was necessary to assess the validity of the control group.

Of other possible factors, the level of carbonmonoxyhaemoglobin and of methaemoglobin must be ruled out because they are low. In addition, the mean corpuscular haemoglobin concentration was similar in the two groups.

Ditzel et al. [15] observed that the level of 2,3-DPG may fluctuate consistently during the day depending on many factors, such as insulin administration and per-

haps physical activity. In order to reduce this uncertainty to a minimum, we studied only hospitalized non-acidotic and non-ketotic patients. In addition, blood samples were taken 3 to 5 hours after the daily insulin dose, in order to reduce any possible effect on the red blood cell metabolic processes, and thus on the level of 2,3-DPG.

The increased oxygen affinity of HbA<sub>1c</sub> has been shown in purified haemoglobin solutions ([Hb] = 0.05 mmol/l, pH 7.0, t = 20 °C, presence of inositol hexaphosphate, low ionic strength) [3], and has been attributed to the low reactivity of this haemoglobin derivative with organic phosphates [16]. This pattern does not occur under the intracellular conditions imposed by this study ([Hb] = 20 mmol/l, pH 7.2, t = 37 °C, high ionic strength, presence of divalent cations and of organic salts). It is possible that spontaneous and rapid dimerization of haemoglobin at low concentrations changes its equilibrium with oxygen, thus altering the physiological conditions under which haemoglobin exists in whole blood [17].

It has been suggested that long-term diabetic complications are mainly caused by tissue hypoxia due to the higher oxygen affinity of glycosylated haemoglobins [8]. Our results suggest that the slightly, although significantly, higher p50 in diabetics may be of little physiological importance, and is however independent of the state of glycosylation of haemoglobin. In addition, there were no significant differences between newly discovered diabetics, and diabetic patients with several years duration of illness.

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