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Human red cell age, oxygen affinity and oxygen transport

Michele Samaja[1], Ermanna Rovida[2], Roberto Motterlini[3], Massimo Tarantola[3], Alessandro Rubinacci[4], and Pietro E. diPrampero[5]

Cattedra di [1]Chimica Biologica and [4]Clinica Ortopedica, Dipartimento di Scienze e Tecnologie Biomediche dell'Università di Milano, Italy, [2]Istituto di Tecnologie Biomediche Avanzate del C.N.R.; [3]Istituto Scientifico San Raffaele, Milano/Italy and [5]Istituto di Biologia dell'Università di Udine, Italy

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Abstract. The [2,3-DPG]/[Hb] ratio and the P_{50} were found to be lower in the 10% denser (old) than in the 10% lighter (young) red blood cell (RBC) fractions (0.57 ± 0.13 *vs* 0.96 ± 0.13 and 23.02 ± 0.85 *vs* 27.47 ± 1.05 Torr, respectively, mean $\pm$ SD, $P < 0.0005$ for both, $n = 6$). The RBC aging processes appear thus to affect the RBC oxygen affinity. However, the [2,3-DPG] changes do not fully explain the drop of P_{50} as measured at constant $[H^+]$, $[CO_2]$ and [HbCO]. It is therefore postulated that an additional factor is involved in the regulation of the oxygen affinity in the ageing RBC. The RBC density in 59 normal individuals matched for age (infants, adult, and aged) and for sex was found to be younger in adult females than in all other groups ($P < 0.0005$), including an age-matched group of pregnant women. Correspondingly, the [2,3-DPG]/[Hb] ratio and the P_{50} are higher in adult females than in adult males (0.92 ± 0.10 *vs* 0.82 ± 0.09, $P < 0.009$, and 29.03 ± 1.07 *vs* 27.72 ± 0.82 Torr, $P < 0.002$, respectively). These data are evaluated in terms of the efficiency of the oxygen transport calculating the circulatory load required to transport a given amount of oxygen to the tissues. The results indicate that the lower oxygen affinity (due to the younger RBC population) in adult females partially compensates for their lower [Hb].

Blood; DPG; Erythrocyte; Human; Oxygen affinity; P_{50}; Pregnancy

The lack of intracellular compartments and of protein biosynthesis, the simple anatomy and physiology, as well as the relative stability of the environment (the blood), are some of the features for which the human red blood cell (RBC) is widely regarded as a model for the study of the cellular aging. In fact, the RBC is irreversibly damaged during its 120 days life span due to the inability to repair from extensive lesions. Therefore, the RBC aging process is characterized by several biochemical changes that are the results of the oxidative damage, such as the metabolic depletion (Seaman *et al.*, 1980), the changes in deformability and shape (Linderkamp and Meiselman, 1982), the decreased activity of the cell enzymes (Kadlubovski and Agutter, 1977), and the decreased affinity

Correspondence address: Michele Samaja, Dipartimento di Scienze e Tecnologie Biomediche, Istituto Scientifico San Raffaele, via Olgettina 60, I-20132 Milano, Italy.

for oxygen (Haidas *et al.*, 1971; Schmidt *et al.*, 1987). In the absence of pathological conditions and hemorrhages, and at constant level of the stressing factors, the average RBC age in an individual is the expression of the equilibrium between the rate of the RBC production by the bone marrow and the rate of the RBC sequestration by the reticulo-endothelial system. Although other indexes are used as well (Clark, 1988), the RBC age is conveniently expressed as a function of the RBC density because the inactivation of membrane Na^+, K^+-ATPase, that is a target of the age-dependent oxidative stress, leads to the RBC shrinking and thus to its higher density.

The proof of the direct correspondence between the RBC age and density is still lacking, but there is very little indication at present that it is incorrect (Clark, 1988). Therefore, we have assumed throughout this paper that the light and dense RBCs are indeed the younger and the older ones, respectively, in a given blood sample.

The biological system represented by the ageing RBC offers a unique opportunity to correlate the main physiological function of the RBC, *i.e.*, the oxygen transport, to the oxidative stress of the circulation led by the cellular aging processes and other factors. Previous studies have shown that the [2,3-DPG]/[Hb] ratio and the P_{50} are lower in the older than in the younger RBC fractions (Haidas *et al.*, 1971; Schmidt *et al.*, 1987), but it is difficult to evaluate if such functional alterations have any physiological meaning for the oxygen transport from lungs to tissues. Indeed, little is known about the RBC average age in normal populations, although it was demonstrated that striking changes are expected under several pathological conditions (Nakashima *et al.*, 1973). In addition, no data are given to determine whether the changes in [2,3-DPG], that is the main effector of the hemoglobin function (Benesch and Benesch, 1967), are quantitatively consistent with the changes in the oxygen affinity, as it should be expected at constant pH, P_{CO_2}, [HbCO], and temperature, that are the other factors that regulate the RBC oxygen affinity.

The aim of this study was: (1) to determine the mechanism underlying the change of the RBC oxygen affinity as a consequence of aging; (2) to explore whether certain subgroups of the healthy human population, such as elderly people, infants, adults, and pregnant women, are characterized by specific RBC age and oxygen affinity patterns; and (3) to estimate by a mathematical model whether the observed changes of the RBC oxygen affinity have a physiological significance in the oxygen transport processes. The results will show that the blood oxygen affinity is finely tuned by the biochemical characteristics of the RBC, and that the response of the RBC to the presence of oxidative factors always tend to restore the basal conditions of oxygen transport.

Materials and methods

Subjects. A total of 75 volunteers of both sexes was examined. All subjects were healthy, with no apparent sign of diseases and no history of metabolic or hematological disorders. Blood was withdrawn from the antecubital vein of the fasting subjects into heparinized tubes, and immediately chilled in an ice bath. Blood was always used within

1 h after withdrawal. The subjects were divided into three age classes: infants, adult, and aged (age range 2–12, 25–35, and 62–85, respectively). In addition, blood was obtained from pregnant women at the 6th to 8th month of pregnancy (age range 23–34).

RBC fractionation. Sixty milliliter of heparinized venous blood from 6 adult males was used. Sodium fluoride was added (final concentration 1 μmol $\cdot$ L^{-1}) to prevent metabolic changes. The cells were fractionated after a reported method (Murphy, 1973) with minor modifications. Blood was centrifuged at 3000 rpm for 10 min at 4 °C to remove plasma that was stored apart. The cells were placed into two 13 × 100 mm tubes (hematocrit > 90%), and centrifuged at 20 000 rpm for 60 min at 30 °C (JA-20 rotor, Beckman Instruments Co, Palo Alto, CA). Finally, the 10% top, young RBC was recovered with a Pasteur pipette, about 80% of the cells was discarded by gentle aspiration with a vacuum pump, and the bottom 10%, old RBC was resuspended in plasma and recovered. The control RBC groups were fresh untreated blood and RBC that were centrifuged but were not fractionated (pool RBC). The top, bottom, and pool RBC fractions were then reconstituted with the native plasma to a 45% hematocrit. To assess the quality of the fractionation, we have measured the mean cellular hemoglobin concentration (MCHC) of the preparations. Preparations with $MCHC_{bottom}/MCHC_{top} < 1.2$ were discarded.

Density gradient profile. Percoll was purchased from Sigma Chemicals, St. Louis, MO, and a stock hyperosmotic solution containing 2.66 mol $\cdot$ L^{-1} NaCl and 0.09 mol $\cdot$ L^{-1} KCl was prepared. Two isotonic solutions were obtained daily mixing 5.5 vols% of the hyperosmotic solution, 65 or 88 vols% of Percoll (to yield densities of 1.090 and 1.120 g $\cdot$ ml^{-1}, respectively), balance with distilled water. Intermediate densities were obtained mixing in appropriate ratios these two solutions. The density gradient profiles were obtained using 10 solutions with increasing density at 0.003 g $\cdot$ ml^{-1} steps. Single point measurements were done using 1.099 and 1.102 g $\cdot$ ml^{-1} densities, at which the difference between the RBC from males and from females is maximal (see Results).

Small centrifuge tubes (4 mm i.d. × 39 mm height) were loaded with 0.15 ml of the isotonic solution with the desired density, and 0.05 ml blood was carefully layered over it. The tubes were centrifuged at 12 000 rpm for 2 min. All operations were performed at 0 °C. At the end of the centrifugation, the blood layers above and below the Percoll were quantitatively removed by a syringe, and were diluted into 5 ml Drabkin's reagent by a precision dispenser (nominal accuracy ± 0.01 ml). The absorbance of the resulting solution was measured in a 1-cm path length cuvette at 540 nm after 60–120 min at room temperature. The absorbance ratio (top)/(top + bottom) yields the fraction of RBC that is lighter than the actual density of the Percoll/isotonic solution.

Determination of the P_{50}. The P_{50} (P_{O_2} at which hemoglobin is half-saturated with oxygen, and a useful index of the RBC oxygen affinity) was determined at three different pH values for each sample. The pH was varied in the range 7.0 to 7.6 with the addition of appropriate amounts of 1 mol $\cdot$ L^{-1} lactic acid or 1 mol $\cdot$ L^{-1} K_2CO_3

to the RBC suspension before the tonometry. The RBC suspension (0.4 ml) was tonometered at 37 °C in flasks (Samaja *et al.*, 1981) that were previously equilibrated at variable known tensions of oxygen and constant tension of carbon dioxide (6.36%, that corresponds to a final P_{CO_2} of 45 Torr). The oxygen saturation and the pH were measured at the end of the tonometry (Samaja and Rovida, 1983, and Instrumentation Laboratory, Paderno Dugnano, Italy, respectively). The P_{50} at pH 7.4 was linearly interpolated from the log P_{50} *vs* pH plots. The P_{50} of the reference blood was calculated using the reported equations (Rovida *et al.*, 1984) at the appropriate [2,3-DPG]/[Hb], pH, P_{CO_2}, and [HbCO].

Prediction of the oxygen transport. Previously described algorithms (Samaja *et al.*, 1986) were used to predict the effect to a change in one of the variables of the oxygen transport on the blood flow or on $P\bar{v}_{O_2}$ that are necessary to maintain the oxygenation at a given metabolic level. This algorithm inputs the parameters of the RBC oxygen affinity, the arterial blood oxygen and carbon dioxide contents, the oxygen uptake, and the respiratory exchange ratio, and outputs the relationship between $P\bar{v}_{O_2}$ and the blood flow that fits all the above variables. At constant $P\bar{v}_{O_2}$ a given alteration of one of the input variables leads either to increase or to decrease the blood flow necessary to sustain the oxygenation. The two conditions are quantitated, and are interpreted to be unfavorable and favorable, respectively, to the peripheral oxygenation. The algorithm can be applied to the whole body (Samaja *et al.*, 1986) as well as to specific organs (Samaja, 1988) provided that the relevant parameters are known or assumed.

Other analyses. Hematological analyses included [Hb] (Drabkin's method), hematocrit (microhematocrit centrifuge), the concentration of HbCO and methemoglobin (metHb) (Rossi-Bernardi *et al.*, 1977) and [2,3-DPG] (Boehringer Biochemia kits), that is here expressed as the molar [2,3-DPG]/[Hb] ratio.

Statistical tests. We have used throughout the parametric Student *t*-test for unpaired data (ANOVA analysis).

Results

The aging process strongly affects the RBC oxygen affinity, because the [2,3-DPG]/[Hb] ratio and the P_{50} are significantly ($P < 0.0005$) lower in the 10% denser (old) than in the 10% lighter (young) RBC fractions (0.57 ± 0.13 *vs* 0.96 ± 0.13 and 23.02 ± 0.85 *vs* 27.47 ± 1.05 Torr, respectively, mean ± SD), N = 6, fig. 1). These data are consistent with previous work (Haidas *et al.*, 1971; Schmidt *et al.*, 1987). The values for the pool and the fresh RBC fractions are similar, indicating that the used protocol does not affect the measured parameters. Indeed, [HbCO] and [metHb] changed slightly following the fractionation from 2.9 ± 0.4% to 3.3 ± 0.6%, and from 0.1 ± 0.1% to 0.9 ± 0.6%, respectively. To assess whether the decreased

[2,3-DPG]/[Hb] ratio is the only determinant of the increased oxygen affinity in the ageing RBC, we have calculated the P_{50} of the reference blood from the equations reported by Rovida *et al.*, 1984, at the actual pH, P_{CO_2}, [2,3-DPG]/[Hb] ratio, temperature and [HbCO]. Then, we have compared the calculated P_{50} to the measured P_{50} (bottom panel of fig. 1). The difference between the two values is virtually zero for the bottom, fresh, and pool RBC fractions, but is significant ($P < 0.005$) for the top RBC fraction.

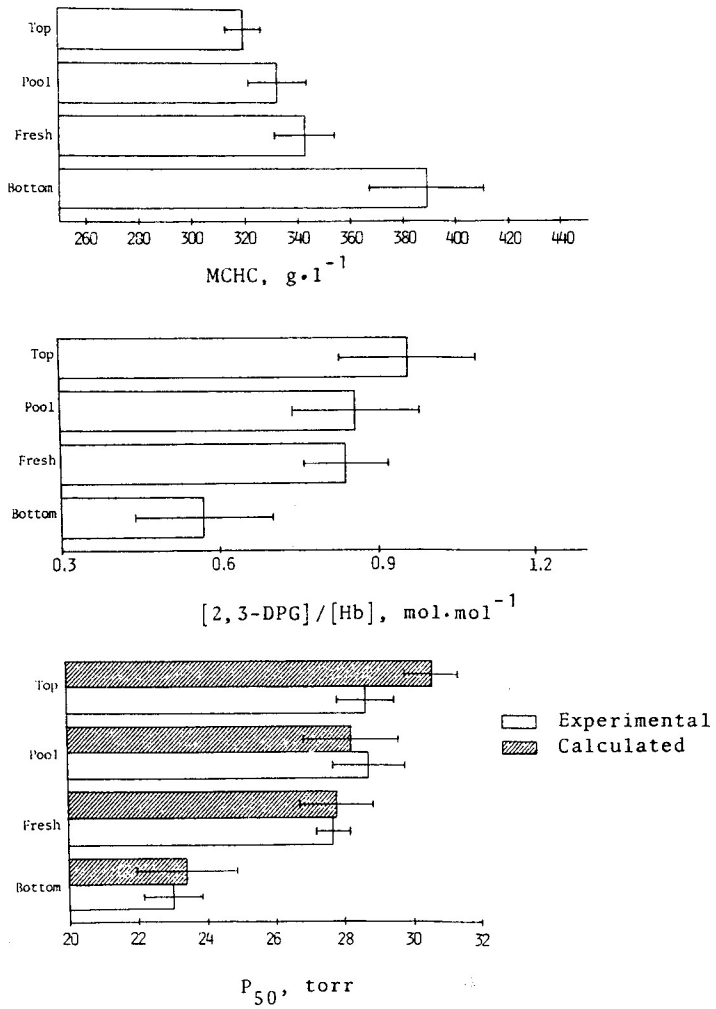

Fig. 1. Mean ± SD, N = 6 for each group, of MCHC, [2,3-DPG]/[Hb], and P_{50} for the top, pool, fresh, and bottom RBC fractions. The bottom panel reports both the experimental P_{50} (plain bars), and the P_{50} calculated from the reported equations (Rovida *et al.*, 1984) (hatched bars). The P_{50} values refer to standard conditions (pH 7.4 and P_{CO_2} = 45 Torr).

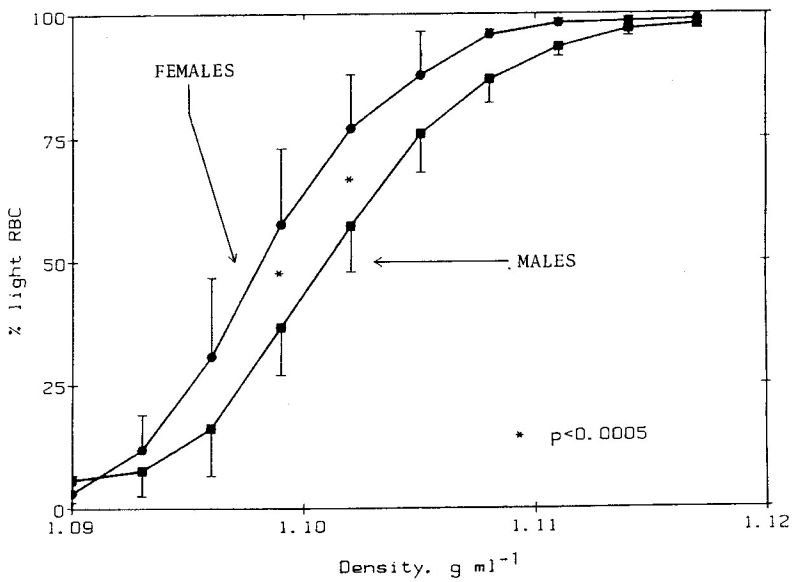

Fig. 2. Density profile of fresh RBC from adult males (N = 9, squares) and adult females (N = 16, circles). The vertical bars represent the SD.

The density profiles of the RBC from 9 adult males and 16 adult females is shown in fig. 2 (percentage of RBC lighter than a given density *vs* the actual density). The difference between the two curves is maximal at d = 1.099 and d = 1.102 $g \cdot ml^{-1}$ ($P < 0.0005$). Table 1 reports the oxygen affinity data obtained in the same subjects. To investigate the reasons for the lower density of the RBC from females, we have determined the percentage of the light, younger RBC fraction from other classes of subjects (table 2). The average value between two separate determinations at d = 1.099 and d = 1.102 $g \cdot ml^{-1}$ is therein reported. Adult females exhibit the youngest RBC with respect to all the other classes of subjects, including the age-matched pregnant women.

The physiological effect of small (1–2 Torr) P_{50} changes on the oxygen transport system was evaluated employing a development of the Fick's equation that uses the actual oxygen equilibrium curves and acid–base state relationships to the arterial and

TABLE 1

[2,3-DPG]/[Hb] ratio and P_{50} (determined at pH 7.4 and P_{CO_2} 45 Torr) in adult males and females.

	N	[2,3-DPG]/[Hb] $mol \cdot mol^{-1}$	P_{50} Torr
Males	9	0.82	27.72
		± 0.09	± 0.82
Females	16	0.92	29.03
		± 0.10	± 1.07
P		< 0.009	< 0.002

TABLE 2

Percent of the light cells in the various classes of subjects. Each determination was performed in duplicate at two Percoll densities (d = 1.099 and 1.102 $g \cdot ml^{-1}$), and the average of the two determinations was calculated. The here reported values are the mean ± SD. NS = not significant ($P > 0.1$); * = significant at the $P < 0.0005$ level (unpaired *t*-test).

	Males				Females		
	N	Age yr	RBC %		N	Age yr	RBC %
Infant	10	7.9 ± 3.0	47.0 ± 13.5	NS	7	6.0 ± 3.0	45.8 ± 16.3
Adult	9	30.1 ± 5.9	46.2 ± 10.7	*	16	27.6 ± 3.0	68.3 * ± 10.4
Pregnant					10	28.2 ± 3.2	49.4 ± 8.1
Aged	7	8.7 ± 3.8	48.8 ± 11.3	NS	10	74.6 ± 9.7	53.9 ns ± 8.2

the venous blood (see Methods and Samaja *et al.*, 1986). Three cases are assumed, and the values for [Hb] ($g \cdot L^{-1}$), [2,3-DPG]/[Hb] ($mol \cdot mol^{-1}$) ratio, and P_{50} (Torr) are respectively: adult males (15.5, 0.82, and 28.0), adult females (13.7, 0.92, and 29.0), and adult females with the same [2,3-DPG]/[Hb] ratio as in males (uncompensated women, 13.7, 0.82, and 28.0). The values for [Hb] and for the [2,3-DPG]/[Hb] ratio are the actually measured values, while the P_{50} values are those calculated (Rovida *et al.*, 1984) and very similar to the measured values reported in table 1. All the other variables are constant (Pa_{O_2} = 95 Torr, Pa_{CO_2} = 40 Torr, base excess = 0 $mEq \cdot L^{-1}$, $\dot{V}_{O_2}$ = 0.35 $L \cdot min^{-1}$, R = 0.8, $P\bar{v}_{O_2}$ = 40 Torr), and the blood flow is the dependent variable (table 3).

TABLE 3

Evaluation of the blood flow necessary to transport a given amount of oxygen in adult males and females using the Fick's equation. Other values are: Pa_{O_2} = 95 Torr, Pa_{CO_2} = 40 Torr, Base excess = 0 $mEq \cdot L^{-1}$, $\dot{V}_{O_2}$ = 0.35 $L \cdot min^{-1}$, R = 0.8; $P\bar{v}_{O_2}$ = 40 Torr. The higher is the blood flow, the heavier is the circulatory load.

	Males	Females	Uncompensated females
[Hb], $g \cdot L^{-1}$	155	137	137
[2,3-DPG]/[Hb], $mol \cdot mol^{-1}$	0.82	0.92	0.82
Measured P_{50}, Torr	27.7	29.0	27.7
Calculated P_{50}, Torr	28.0	29.0	28.0
Blood flow, $L \cdot min^{-1}$	6.34	6.80	7.12

Discussion

The RBC aging process and the oxygen affinity. The RBC fractionation according to density is fast, does not require the removal of leukocytes and platelets, and uses native plasma to generate the density gradient avoiding the use of extraneous compounds that may interfere with the oxygen affinity. These features are also supported by the observed similarity of the hematological values between the pool and the fresh RBC fractions (fig. 1).

The human RBC age-induced metabolic changes are reflected by the increased MCHC, perhaps the most reliable index of the RBC age, and by significant changes of the oxygen affinity, consistently with previous findings (Haidas *et al.*, 1971; Schmidt *et al.*, 1987). Furthermore, the [2,3-DPG]/[Hb] ratio also decreases markedly with age nearly halving from the top to the bottom RBC fraction (fig. 1). Assuming a constant RBC hemoglobin content (MCH) throughout the RBC life span (*i.e.*, there is a loss of water but not of hemoglobin from the RBC), the latter finding can be attributed to the dehydration of the RBC. Indeed, at constant [2,3-DPG] in the cell water, the dehydration and the consequent cell shrinking (volume reduction) would lead '*per se*' to a decreased [2,3-DPG]/[Hb] ratio. A metabolic depression of the RBC (due to the age-dependent enzyme inactivation) may induce an even larger fall of the [2,3-DPG]/[Hb] ratio than accounted for the RBC dehydration only. The considerations that follow, summarized in table 4, show that both RBC dehydration and metabolic depression contribute about equally to the changes of the [2,3-DPG]/[Hb] ratio in the ageing RBC.

We have assumed that: (1) the MCH is constant (29×10^{-12} g/cell) throughout the RBC life; (2) the specific volume of hemoglobin (V_{Hb}) is 0.75 fl · pg^{-1} (Kunitz *et al.*, 1933/34); and (3) the RBC volume (MCV) falls from 90 fl to 74 fl during aging, as a result of dehydration. On the bases of these assumptions, the water content per cell (V_w) can be calculated as $V_w = MCV - (V_{Hb} \times MCH)$. The 2,3-DPG content in the RBC can also be calculated from the assumed MCH and the observed [2,3-DPG]/[Hb] values. Inspection of table 4 (bottom/top values) shows that the decrease of the water

TABLE 4

Calculation of [2,3-DPG], expressed as mmol · L^{-1} RBC water in the top and bottom RBC fractions. The assumptions are explained in the text.

	Top	Bottom	Bottom/top
[2,3-DPG]/[Hb], mol · mol^{-1}	0.96	0.57	0.59
MCHC, g · L^{-1}	320	390	1.24
MCH, pg/cell	29	29	1.00
MCV, fl/cell	90	74	0.82
V_w, fl/cell	68	52	0.76
[2,3-DPG], fmol/cell	0.43	0.26	0.60
[2,3-DPG], mmol/(L cell H_2O)	6.3	4.9	0.78

volume is smaller than the corresponding decrease of the 2,3-DPG content. As a consequence, the concentration of 2,3-DPG in the cell water must also be decreased. Thus, these considerations suggest that the decreased [2,3-DPG]/[Hb] ratio can be attributed about equally to both the water loss from the RBC and the metabolic depression of the RBC. The former is due to the depression of Na^+, K^+-ATPase, and the latter to the depression of the metabolic pathways leading to 2,3-DPG. These considerations, however, do not change the figures concerning the RBC oxygen affinity, because in this case the ratio [2,3-DPG]/[Hb] only is relevant.

The consistency of the decrease of the [2,3-DPG]/[Hb] ratio with the decrease of the P_{50} was assessed comparing the measured P_{50} to the P_{50} calculated for a reference human blood under the same conditions of temperature, pH, P_{CO_2}, [2,3-DPG]/[Hb], and [HbCO] (Rovida *et al.*, 1984). In this approach, we assume that the value of the P_{50} is fully described by the mentioned factors, and that no other factors are present. Therefore, if the difference between the two values is significant, then the presence of a factor other than the mentioned ones is to be postulated. It appears (bottom panel of fig. 1) that the calculated P_{50} satisfactorily matches the measured one for the bottom RBC fraction and for the control RBC groups, but not for the top RBC fraction, indicating the presence, in the top, young RBC only, of a factor that increases the hemoglobin oxygen affinity, and that is not 2,3-DPG, H^+, CO_2, nor HbCO, because the effect of these compounds is already included in the equations needed to calculate the P_{50}. The possible role of ATP and of glycated hemoglobins is ruled out because the effects driven by these factors run in the direction opposite to that observed. Possibly, membrane Na^+, K^+-ATPase plays a significant role in the regulation of the oxygen affinity in the ageing RBC by altering the intracellular concentrations of Na^+ and K^+, that in turn alter the intracellular pH, or interfere with both the hemoglobin oxygen affinity and the 2,3-DPG complex with Hb.

The RBC density profiles. The average RBC from adult females is younger than that of the age-matched males. No difference in the RBC density distribution was detected among the classes of males, nor between the males and the infant and the aged females. There are mainly two explanations for this observation: (1) the oxidative factors in the blood of adult females are less than in the other subjects, possibly because of the protection afforded by the higher hormone levels in the female blood (but in pregnant women the RBC density is the same as in males, in spite of their still higher hormone level); and (2) the life span of the RBC in adult females is shorter than that of the other subjects, possibly because of the enhanced erythropoiesis stimulated by the periodical blood losses in menstruating women. Whatever the explanation and the meaning for the younger RBC in adult menstruating females, the higher [2,3-DPG]/[Hb] ratio, and hence the higher P_{50} (table 1), is well correlated to the younger RBC population in women.

The RBC aging process and the oxygen transport. It is generally agreed that a decreased RBC oxygen affinity implies a more efficient oxygen unloading to tissues, and thus the

oxygen transport seems more efficient in adult females than in the other groups. We have predicted the size of the advantage simulating the oxygen transport as an integrated index of the parameters that are described in the well known Fick's equation. The circulatory load is conveniently expressed by the blood flow necessary to transport a given amount of oxygen at constant values for the other parameters, *i.e.*, arterial and venous gasses, [2,3-DPG]/[Hb], [Hb], the oxygen uptake, and the respiratory exchange ratio (Samaja *et al.*, 1986). The data reported in table 3 were calculated using actual [Hb] in males and females, and the [2,3-DPG]/[Hb] values reported in table 1. In adult females, the advantages of the decreased RBC oxygen affinity are almost completely offset by their decreased [Hb]. Indeed, should females have the same [2,3-DPG]/[Hb] as males, the circulatory load should increase by more than 10% (see 'uncompensated women'). Thus it seems that the younger RBC population with enhanced oxygen transport in females is a physiological response to their lower [Hb].

Conclusion. The ageing RBC is a valuable model to correlate the cellular function to the circulatory oxidative stress. Our data are in agreement with other work on acclimatization to moderate (Mairbaurl *et al.*, 1986) and high altitudes (Winslow *et al.*, 1989) indicating that the erythropoiesis may be increased upon adaptation, and that the average RBC population may become younger with enhanced oxygen transport characteristics under the hypoxic stimulus. Our findings also indicate that the human blood is not homogeneous with respect to the oxygen transport and likely to other functions, such as for example the active transport of calcium across the RBC membrane (Samaja *et al.*, 1989). The implications of such heterogeneities in the physiology of the oxygen transport are still to be evaluated, but the long-accepted observation that females are normally less subjected to cardiovascular diseases than males (Bush *et al.*, 1987) well correlates to the data reported here.

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