THE EFFECT OF *IN VIVO* AGING OF NORMAL HUMAN ERYTHROCYTES AND ERYTHROCYTE MACROMOLECULES UPON OXYHEMOGLOBIN DISSOCIATION *

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Physical, chemical and metabolic alterations are known to occur during the *in vivo* aging of the normal human erythrocyte (1–16), including changes of hemoglobin molecular structure (14–16). There have been no reports of the influence of erythrocyte age upon the erythrocyte's function of oxygen transport. Our purpose has been to determine whether a change in oxyhemoglobin dissociation occurs with aging of the normal human erythrocyte.

Any observed differences of oxyhemoglobin dissociation characteristics of old and young erythrocytes would require consideration of an age-dependent alteration of erythrocyte: metabolic activity, cell membrane function, electrolyte composition, small molecules and metabolites, and macromolecules, including hemoglobin itself. The plan of this study was designed to consider these possibilities by studies of hemoglobin: *1*) contained within intact viable erythrocytes, and *2*) freed of erythrocyte influence by hemolysis and subsequent dialysis.

METHODS

It has been well established that the aging erythrocyte increases in density (5, 7, 10, 11). Following conventional centrifugation of blood containing Fe^{59}-labeled hemoglobin, several investigators have consistently observed that the top layers of the packed red cells contain cells of younger than average mean cell age, whereas the bottom layers contain cells of older than average mean cell age (5, 7, 10, 11). Ultracentrifugation modifies this technique only by applying a much greater gravitational force thereby improving the separation of cells according to age (11). Ultracentrifuga-

tion is used in this study as the means of separating older cells from younger cells.

Oxyhemoglobin saturation was measured at an arbitrarily selected Po_2 of 30 mm Hg and Pco_2 of 42 mm Hg in young and old intact erythrocytes, and in dialyzed hemolysates of young and old erythrocytes.

Intact erythrocytes. Blood samples were taken from the arm veins of 10 young, healthy, male medical students and interns. Heparin was used as an anticoagulant. Each sample was immediately cooled to 0° to 4° C and maintained at this temperature, except where otherwise specified. The buffy coat was removed following brief preliminary centrifugation at 1,200 G (2,600 rpm, radius 16 cm). Ultracentrifugation for 2 hours at 67,-000 G at the tip (22,500 rpm, radius 11.9 cm) was carried out as described by Rigas and Koler (11). The Lusteroid containers were cut transversely and three equal layers of packed erythrocytes were separated, consisting of top, middle and bottom thirds; the middle third was discarded. The top and bottom thirds will be subsequently referred to as top layer cells and bottom layer cells, each nonspecifically as a cell layer. The cell layers were washed four times with separate samples of the subject's plasma, thereby removing all free hemoglobin. The packed top and bottom cell layers were separately suspended in samples of the subject's plasma, providing in each a hemoglobin concentration approximately equal to that of the original whole blood sample.

Equilibration of the cell layers with gas mixtures was accomplished in a modification of the open-circuit tonometer system of Björk and Hilty (17). The gas mixtures, prepared and stored under pressure in gas cylinders, were analyzed for their fractional compositions of oxygen and carbon dioxide by the Scholander microvolumetric gas analyzer (18). The temperature of the water bath was kept at 37.16° C ($\pm 0.001°$ C). The gas mixture was finally ejected from the tonometer system at a measured depth under water. By varying this depth each day by an amount sufficient to compensate for fluctuations of barometric pressure, it was possible to maintain constancy of Po_2 and Pco_2 in the tonometer chamber. Under the conditions of this technique, equilibration of cell layer and gas mixture was always complete within 20 minutes.

The temperature of 37.16° C during equilibration permitted continuous glycolysis with accumulation of acid metabolites. The increasing acidity during the equili-

bration period by itself decreased oxygen and carbon dioxide contents of the cell layers. (The use of glycolysis-inhibiting agents, such as sodium fluoride and diiodoacetate, always resulted in excessive hemolysis. By permitting glycolysis to occur, hemolysis was always avoided.) At a P_{O_2} of 30 mm Hg and P_{CO_2} of 42 mm Hg, the simultaneous decrease of oxygen and carbon dioxide contents was curvilinear as shown in Figure 1. During equilibration at a P_{O_2} of 147 mm Hg and P_{CO_2} of 42 mm Hg for the measurement of oxygen capacity, carbon dioxide content decreased without alteration of oxygen content.

In order to assess the relation of blood oxyhemoglobin to carbon dioxide content under changing conditions of acidity, each cell layer was separated into three 2 to 3 ml aliquots, and each in turn was equilibrated in the tonometer chamber. Two of the aliquots were equilibrated at a P_{O_2} of 30 mm Hg and P_{CO_2} of 42 mm Hg, one for 20 minutes and the other for 60 minutes. The third aliquot was equilibrated at a P_{O_2} of 147 mm Hg and P_{CO_2} of 42 mm Hg for 20 minutes. Following equilibration, a small syringe moistened with heparin was attached to the aspirating needle. The tonometer gas was admitted to and expelled from the syringe six times. The equilibrated aliquots were then aspirated into the syringe and promptly analyzed for

their oxygen and carbon dioxide contents by the Van Slyke-Neill manometric technique (19). Dissolved oxygen content was calculated (20) and subtracted from the total oxygen content to determine oxyhemoglobin content. The oxyhemoglobin content of the aliquot equilibrated at a P_{O_2} of 147 mm Hg was the oxygen capacity of its cell layer. The oxyhemoglobin percentage saturations of the aliquots equilibrated at a P_{O_2} of 30 mm Hg were then calculated. All studies were completed within 9 hours after collection of the blood sample.

Dialyzed clarified hemolysates. Blood samples from four young, healthy, male medical students and interns were processed by ultracentrifugation as described in the previous section. Each top and bottom cell layer was washed three or four times with isotonic saline. The cell layers were then hemolyzed by the addition of 2 or 3 vol of deionized water per vol of packed cells and were subsequently subjected to repeated rapid freezing and thawing. Each hemolysate prepared from the top layer cells or bottom layer cells will be subsequently referred to as a top layer hemolysate or bottom layer hemolysate, each nonspecifically as a cell layer hemolysate. The cell layer hemolysates were dialyzed against deionized water for 36 to 48 hours. Ultracentrifugation for 4 to 5 hours at 144,700 G (40,000 rpm, radius 8.1 cm) removed the stroma thereby clarifying the cell layer

TABLE I

Oxyhemoglobin and carbon dioxide contents of ultracentrifuged layers of erythrocytes after equilibration with gas mixtures containing a P_{CO_2} of 42 mm Hg

P_{O_2} (mm Hg)	30						147	
Equilibration time (min)	20			60			20	
Subject	CO₂	HbO₂	HbO₂	CO₂	HbO₂	HbO₂	CO₂	HbO₂
	vol %		*% sat.*	*vol %*		*% sat.*	*vol %*	
Top layer								
M.F.	49.77	10.94	58.53	48.26	9.80	52.43	46.98	18.69
J.M.	51.35	10.93	60.79	48.95	10.00	55.62	49.89	17.98
J.A.	50.51	10.79	57.49	47.73	9.53	50.77	48.25	18.77
R.W.	51.12	10.74	58.85	48.38	9.54	52.27	47.91	18.25
A.Ki.	53.39	11.73	65.49	50.91	10.56	58.96	50.33	17.91
R.L.	52.91	10.41	57.04	50.83	9.61	52.66	50.21	18.25
L.T.	53.16	9.88	59.09	51.39	9.05	54.13	49.84	16.72
F.F	53.43	10.60	61.24	52.13	9.75	56.33	52.03	17.31
C.C.	53.38	11.64	59.91	49.80	10.40	53.53	49.61	19.43
A.Ke.	51.29	12.09	60.85	49.20	11.36	57.17	50.75	19.87
Mean	52.05	10.98	59.91	49.76	9.96	54.37	49.58	18.32
Bottom layer								
M.F.	49.95	10.99	60.52	47.64	10.17	56.00	47.94	18.16
J.M.	52.92	11.99	67.21	51.14	11.39	63.85	50.36	17.84
J.A.	50.28	11.32	64.25	49.02	10.27	58.29	48.63	17.62
R.W.	51.71	12.21	61.64	49.49	11.47	57.90	49.81	19.81
A.Ki.	51.74	12.36	68.33	50.27	11.52	63.68	49.91	18.09
R.L.	53.73	11.12	63.98	52.27	10.51	60.47	52.97	17.38
L.T.	52.42	10.71	64.48	51.00	10.04	60.45	50.89	16.61
F.F.	55.66	11.77	67.53	53.47	10.76	61.73	53.86	17.43
C.C.	53.02	11.82	64.48	50.65	10.79	58.87	50.38	18.33
A.Ke.	51.81	12.57	68.06	51.10	12.10	65.51	48.81	18.47
Mean	52.32	11.69	65.01	50.60	10.90	60.65	50.36	17.97

hemolysate. Further ultracentrifugation at 144,700 G concentrated the hemoglobin to different extents in the different cell layer hemolysates.

Each cell layer hemolysate was divided into two 2 to 3 ml aliquots, and each of these was subjected to 20 minutes' equilibration. (Here, it was unnecessary to vary the duration of equilibration because no detectable glycolysis occurred.) One aliquot was equilibrated at a P_{O_2} of 30 mm Hg and P_{CO_2} of 42 mm Hg, the other at a P_{O_2} of 147 mm Hg and P_{CO_2} of 42 mm Hg. Oxygen and carbon dioxide contents were determined and further calculations were performed as in the previous section.

The pH of each aliquot equilibrated at a P_{O_2} of 147 mm Hg and P_{CO_2} of 42 mm Hg was determined in two studies using a Cambridge research model pH meter with temperature controlled at 37° C. In one study, spectral analyses for methemoglobin were done using the Cary recording spectrophotometer. In another, inactive hemoglobin (primarily methemoglobin) contents were calculated by subtracting active hemoglobin contents (oxygen capacity/1.34) from total hemoglobin contents, determined by the cyanmethemoglobin method (21). All studies were completed within 4 days after collection of the blood sample.

RESULTS

Intact erythrocytes. Oxyhemoglobin and carbon dioxide contents of each cell layer are listed in Table I. For each period of equilibration, the carbon dioxide contents of top layer cells were generally lower than those of corresponding bottom layer cells, presumably due entirely to the greater rate of acid accumulation in the metabolically more active young cells (4, 5). Oxyhemoglobin saturations of top layer cells were always lower than those of corresponding bottom layer cells. Since there was unequal metabolic activity in the top and bottom cell layers, it became necessary to assess the role of glycolysis as a possible explanation for the different oxyhemoglobin saturations.

The raw data were analyzed by multiple regression using each oxyhemoglobin and carbon dioxide content of each cell layer as an independent variable for discrimination in comparing the oxyhemoglobin contents of corresponding top and bottom cell layers (22). This statistical treatment demonstrated that the different rates of glycolysis reflected in the different carbon dioxide contents could not account for all of this difference of oxyhemoglobin saturation. (Experimental validation of this is shown in the subsequent studies of dialyzed hemolysates.) The bottom layer cells had a significantly $(p < 0.05)$

SAMPLE OF UNSEPARATED ULTRACENTRIFUGED BLOOD. BLOOD GAS CONTENTS-OXYGEN PRESSURE 30 mm. Hg. AND CARBON DIOXIDE PRESSURE 42 mm. Hg.
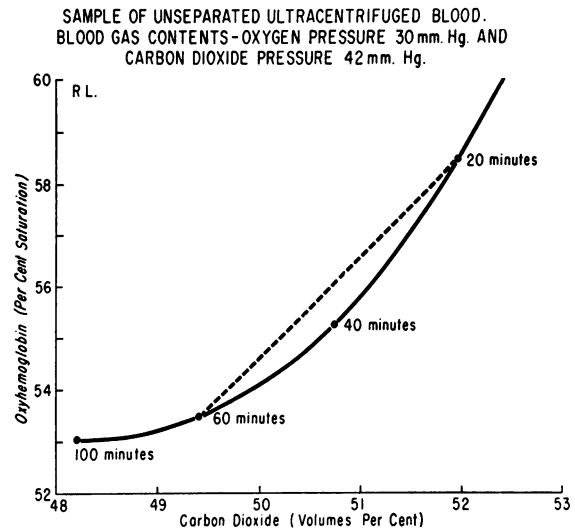
FIG. 1. THE RELATIONSHIP OF EQUILIBRATION TIME TO BLOOD OXYHEMOGLOBIN SATURATIONS AND CARBON DIOXIDE CONTENTS.

greater oxyhemoglobin saturation than top layer cells under these conditions of equilibration.

Figure 1 demonstrates that the relation of oxyhemoglobin saturation to carbon dioxide content during continuing glycolysis is virtually linear between the twentieth and sixtieth minutes of equilibration. The mean values of the ten studies of intact cells are shown graphically in Figure 2. Straight lines have been assumed to represent the approximate pathway taken by the top and bottom cell layers in terms of oxyhemoglobin saturation and carbon dioxide content between the twentieth and sixtieth minutes of equilibration. Oxyhemoglobin saturations of top and bottom cell layers can be compared at any shared intercept of carbon dioxide content, thereby correcting for unequal metabolic activity. The oxyhemoglobin saturation difference between the mean top and bottom cell layer pathways is 4.35 per cent.

Dialyzed clarified hemolysates. The oxyhemoglobin and carbon dioxide contents, together with selected pH measurements, are listed in Table II. The bottom layer hemolysates had a 4.71 per cent greater mean oxyhemoglobin saturation than the top layer hemolysates. The variable oxygen capacities (reflecting variable hemoglobin concentrations) did not affect oxyhemoglobin saturation. The differences between the oxyhemoglobin saturations of corresponding top and bottom cell layer hemolysates were analyzed statis-
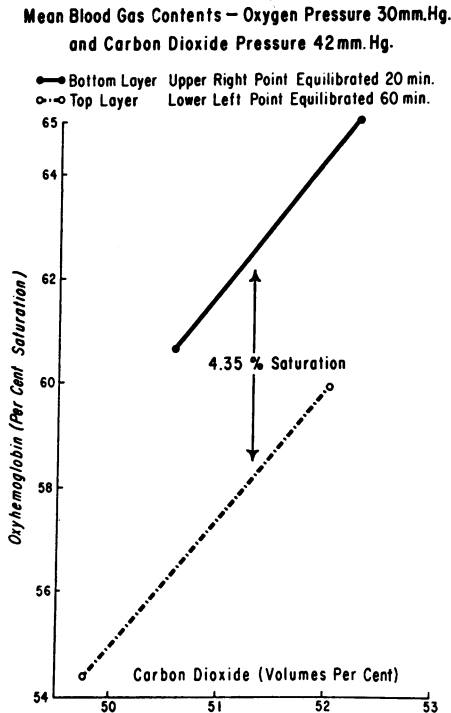
Mean Blood Gas Contents — Oxygen Pressure 30mm.Hg.
and Carbon Dioxide Pressure 42mm. Hg.

•—• Bottom Layer   Upper Right Point   Equilibrated 20 min.
o--o Top Layer   Lower Left Point   Equilibrated 60 min.

Fig. 2. THE MEANS OF THE OXYHEMOGLOBIN SATURA-
TIONS AND CARBON DIOXIDE CONTENTS OF ALL TEN STUD-
IES OF TOP AND BOTTOM LAYERS OF ULTRACENTRIFUGED
ERYTHROCYTES.

tically (22). The bottom layer hemolysates had a
significantly (p < 0.05) greater oxyhemoglobin
saturation than top layer hemolysates under these
conditions of equilibration.

The pH values determined in two studies at a
P_{O_2} of 147 mm Hg and P_{CO_2} of 42 mm Hg were
essentially the same in corresponding top and bot-
tom cell layer hemolysates. Methemoglobin was
not detected in either top or bottom cell layer he-

TABLE II

*Blood gas contents and pH of dialyzed hemolysates of ultra-
centrifuged layers of erythrocytes after 20 minutes' equili-
bration with gas mixtures containing a P_{CO_2} of 42 mm Hg*

P_{O_2} (mm Hg) Subject	30			147		
	CO₂	HbO₂	HbO₂	CO₂	HbO₂	pH
	vol %		% sat.	vol %		
Top layer						
M.F.	10.50	4.40	52.01	10.17	8.46	
A.Ki.	14.33	9.22	62.94	13.81	14.65	6.70
C.C.	10.49	5.02	50.60	10.18	9.92	6.59
P.C.	6.28	1.52	48.25	6.48	3.15	
Bottom layer						
M.F.	10.73	5.13	53.22	11.02	9.64	
A.Ki.	14.35	10.94	67.41	13.72	16.23	6.71
C.C.	9.85	5.13	56.19	9.07	9.13	6.58
P.C.	8.02	3.03	55.80	8.02	5.43	

TABLE III

*Comparison of total and active hemoglobin of dialyzed hemol-
ysates of ultracentrifuged layers of erythrocytes of
Subject C.C.*

Layer	Total hemo-globin	Active hemo-globin	Inactive hemo-globin	Oxygen capacity total hemo-globin
	g %	g %	g %	ml/g
Top	7.6	7.40	+0.20	1.31
Middle	7.1	7.22	−0.12	1.36
Bottom	6.7	6.77	−0.07	1.36

molysates, in the two studies performed, either
by spectrophotometry or by determination of in-
active hemoglobin (see Table III). There was
no apparent alteration with age of the normal oxy-
gen capacity-hemoglobin ratio (normally 1.34
ml O_2 per gram Hb).

DISCUSSION

Erythrocyte aging has been studied by the
techniques of serial osmotic hemolysis (12, 13);
differential agglutination, sparing transfused do-
nor cells (23–25); and centrifugation (5, 7, 10,
11). It has been established that numerous
changes occur during the life span of the mature
circulating erythrocyte. Enzymatic activity (1–6)
and anaerobic glycolysis (4, 5) decrease. Po-
tassium concentration decreases with a rise in so-
dium concentration (5, 7). A controversy exists
(8, 9) as to whether methemoglobin concen-
tration may increase. The minor hemoglobin
components, A_3 and A_1, increase with a reduc-
tion of major hemoglobin A (14, 15). Osmotic
fragility increases (12, 13). Erythrocyte lipid
content decreases, causing diminished cell size
and increased cell density (5, 7).

At a P_{O_2} of 30 mm Hg and P_{CO_2} of 42 mm Hg,
the older intact erythrocytes have an average of
4.35 per cent greater oxyhemoglobin saturation
than the younger erythrocytes. The difference
between the old and young cells at one point on
the oxyhemoglobin dissociation curve confirms
their dissimilarity.

Many factors act upon hemoglobin to influence
its property of reversible combination with oxy-
gen: pH (26), CO_2 (27), temperature (28), elec-
trolyte environment (29–32), cell thickness (33),
carbonic anhydrase (34), ascorbic acid (34), sulf-
hydryl groups of cysteine and glutathione (35),

sulfhydryl groups of hemoglobin (36–38), the spatial arrangement of the four heme groups within the hemoglobin molecule (39, 40), and the presence of the inactive forms of hemoglobin, carbonmonoxyhemoglobin (41) and methemoglobin (42).

It was not clear, however, from the studies of viable, intact cells whether these or other factors were responsible for the observed change in oxyhemoglobin dissociation with age. Hemolysis followed by dialysis and removal of the stroma destroyed not only the erythrocyte membrane but also its metabolism by effectively removing all ions and metabolic participants. The residue contained only the soluble erythrocyte macromolecules, principally hemoglobin. The pH of the oxygen-saturated hemolysates was essentially that of isoelectric oxyhemoglobin. Oxyhemoglobin saturation was significantly greater in the dialyzed hemolysates of old cells than in those of young cells and was comparable in magnitude to that observed between old and young intact cells. It was concluded, therefore, that the age-dependent dissimilarity in oxyhemoglobin saturations of intact cells and their dialyzed hemolysates was attributable to alterations of erythrocyte macromolecules.

Such a technique does not exclude the possible influence of methemoglobin. According to Darling and Roughton, a 14 per cent (of total hemoglobin) concentration of methemoglobin is necessary to effect a 4 to 5 per cent increase of oxyhemoglobin saturation in hemolysates in this part of the oxyhemoglobin dissociation curve (42). Waller, Schlegel, Müller and Löhr found a methemoglobin concentration of 8 per cent of total hemoglobin in erythrocytes surviving 80 days after transfusion (8). Betke, Baltz, Kleihauer and Scholz, however, measured mean methemoglobin concentrations of only 0.6 per cent of total hemoglobin in the top and bottom layers of centrifuged erythrocytes (9). Our failure to detect methemoglobin makes it unlikely that the observed differences of oxyhemoglobin saturation could be explained on this basis.

The results of Meyering, Israels, Sebens and Huisman (15) demonstrate a partial conversion with age of normal hemoglobin A_0 to the minor hemoglobin A_1. They observed no difference, however, in the oxygen affinity of these two hemoglobins which were prepared from erythrocytes not separated according to age. The high oxyhemoglobin affinity of hemoglobin A_2 (15) does not explain our results, since this hemoglobin did not increase as erythrocytes aged.

The macromolecules of dialyzed, stroma-free hemolysates consist principally of hemoglobin, about 99.2 per cent by weight (43). Other nondialyzable components include enzymes whose alterations with age have been described. The substrates upon which the enzymes normally act, however, have been removed by dialysis. Other macromolecules are unknown and, if present, probably exist only in minute amounts. In a preliminary study of major hemoglobin A isolated by starch grain electrophoresis, its oxyhemoglobin saturation at a P_{O_2} of 30 mm Hg and P_{CO_2} of 42 mm Hg was greater in a sample prepared from the bottom layer hemolysate than in that prepared from the top layer hemolysate. These facts together indicate that a macromolecular alteration has probably occurred in the hemoglobin molecule itself.

This study has demonstrated an alteration of oxygen dissociation properties of normal hemoglobin as a result of *in vivo* erythrocyte aging. Such a change in the function of a normal protein presumably reflects an age-dependent alteration of its structure. In this instance, however, altered structure is not the result of proteolysis since aged hemoglobin retains a normal oxygen capacity. This may be secondary to an *in vivo* erythrocyte metabolic alteration or an independent and spontaneous molecular change. This study, together with the previously reported molecular alterations of hemoglobin (14–16) and of lens proteins (44) with age, supports the concept that proteins in their physiological environment may undergo with age a molecular alteration other than proteolysis, impairing the function and/or viability of the cells (16, 45).

Further studies are now in progress to define more fully the effect of cell age upon the oxyhemoglobin dissociation curve and to determine the nature of the postulated hemoglobin molecular change.

SUMMARY

1. Oxyhemoglobin dissociation properties of old and young normal human erythrocytes and their

dialyzed hemolysates have been compared. The different densities of old and young erythrocytes permitted their separation by ultracentrifugation.

2. Old and young intact erythrocytes obtained from ten normal subjects were separately equilibrated at 37.16° C with a P_{O_2} of 30 mm Hg and P_{CO_2} of 42 mm Hg. Old erythrocytes had a significantly greater oxyhemoglobin saturation than young erythrocytes ($p < 0.05$).

3. Dialyzed and clarified hemolysates of old and young erythrocytes from four normal subjects were separately equilibrated at 37.16° C with a P_{O_2} of 30 mm Hg and P_{CO_2} of 42 mm Hg. (Hemolysates dialyzed against deionized water contain only erythrocyte macromolecules.) The dialyzed hemolysates of old erythrocytes had a significantly greater oxyhemoglobin saturation than the dialyzed hemolysates of young erythrocytes ($p < 0.05$). It was concluded that the difference in oxyhemoglobin dissociation of young and old intact erythrocytes is attributable to alteration of erythrocyte macromolecules.

4. The age-dependent alteration in the function of normal hemoglobin is probably due to an alteration within its molecule. This supports the hypothesis that normal proteins may change in structure as a result of aging.

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