UR-3490- 1 5 4 3

TITLE: "Aged" (Dense) Circulating Red Cells Contain Normal Concentrations of ATP

We have applied a newly-developed technique for determination of the ATP content of individual red cells to the densest, and hence presumably the oldest (1), cells from normal human blood. We find that these cells contain normal concentrations of ATP, although the net content of ATP is decreased. The essence of the technique is suspension of red cells in autologous plasma containing luciferin and luciferase, lysis of the cells with a pulse from a laser, and counting of the photoemissions resulting from reaction of the released ATP with the luciferase. Details of the technique are to be published elsewhere (2); preliminary accounts of the technique have been published (3,4).

The average ATP content of red cells apparently decreases with cell age (5), and it is clear that decreased ATP concentration correlates with loss of viability of blood during storage in the blood bank (6). Hence, there has been a recurrent suspicion that decreased ATP concentration might be involved in destruction of senescent red cells, despite some contrary evidence (7,8).

We separated the densest 0.1% of the red cells from samples of normal blood by a two-step procedure. In the first step, blood was centrifuged through step gradients of Hypaque and NaCl. Densities were adjusted to obtain the densest or least dense 1-3% of the cells (9). These cells were then sealed into 1.2mm plastic tubing (inner diameter; PE-190, Clay-Adams) at high hematocrit and centrifuged at 27,000g for 1 hr at 25°C (10). The densest or lightest 1-2% of such cells were used for these experiments, and were easily obtained by slicing the tubing at an appropriate level. As a control, cells were processed in the same manner but were re-mixed after each step.

DISCLAIMER

Uncorrected ATP contents for dense and normal cells are shown in Fig. 1. The average number of counts and total ATP contents for dense cells and normal cells are significantly different (Table 1), but must be corrected to obtain concentrations. The primary correction is for size: the dense cells are significantly smaller than normal cells (Table 1). In addition, the dense cells are dense in part because they have increased cellular hemoglobin levels (Table 1), implying decreased intracellular water.

The ATP content was obtained from the mean photon count by the calibration curve of Fig. 2. This curve was obtained by inhibiting ATP resynthesis of unfractionated red cells with iodoacetamide, adding inosine, and measuring ATP content (by a "bulk" method) and photon counts at various times. (See Ref. 2 for details). The calibration curve is quite linear but does not pass through the origin. We do not understand why it does not, and hence feel that the absolute value of the ATP contents reported here shoud be treated with caution. The effect of correction for the non-zero intercept is to increase the apparent ATP content of the dense cells relative to the normal cells by about 10%. However, the dense cells appear to have about the same ATP concentration as unfractionated cells whether or not this correction is applied.

The results presented above could have been obtained without the use of the laser lysis-luciferin technique, since enough cells are present even at the end of the second stage of fractionation for analysis of ATP content by conventional methods if pressed to the limit. The novel datum obtained by the laser technique is the distribution of ATP content in the population of cells (Fig. 1). The width of this distribution in dense cells is essentially identical with that in control cells, and this means that there is no evidence for a relative increase in cells with a greatly diminished ATP content or

concentration. One would expect to observe a marked tailing in the left side of the curve for "dense" cells in Fig. 1 if cells either decreased in ATP concentration as they aged (and became more dense), or if cells underwent a sudden decline of ATP during the last 10% or more of whatever residual lifespan is ascribed to the dense fraction. If our fractionation were perfect, then these cells would represent about 2% x 5% = 0.1% of the population. Since about 1% of the population is removed from circulation each day, the dense cells represent only the "quota" for the next day even if the fractionation is only 1/10 as efficient as it should be.

Hence these data appear to disprove the otherwise plausible hypothesis of Lichtman (5) that red cells decline exponentially in ATP content as they age, by one of the suggested (5) tests of this hypothesis. The data suggest an alternative hypothesis: red cells maintain an approximately constant concentration of ATP as they age, and red cell destruction is caused by factors other than cellular ATP.

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Table 1. Hematologic Parameters and ATP Content of Fractionated Red Cells.

Parameters	Densest Cells	Control Cells	Lightest Cells
Number of cells[a]	60	32	62
Mean photon counts±S.E.	48,000±3,100	70,00[illegible]00	81,000±2,400
Mean cell volume (femtoliters)[b]	70.2	89.1	105.3
Mean hemoglobin/cell (g/liter cells)[c]	432	344	335
ATP content (mol/cell, $x10^{17}$)[d]	9.4	12.6	14.3
ATP concentration, mmol/l cells	1.34	1.41	1.36
ATP concentration, mmol/l cell water[e]	1.96	1.98	1.80

a: Measured by the single cell technique.

b: Measured on a Celloscope.

c: Hemoglobin measured according to (11).

d: Using calibration curve (Fig. 2).

e: Calculation of hemoglobin volume using a partial specific volume of 0.73, and subtraction of that volume from cell volume.

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FIGURE LEGENDS

FIGURE 1: Distribution of Photon Counts and Cell-Counter Channels ("Volume") in Fractionated Erythrocytes.

The relative frequencies of photon counts and of channel number for erythrocytes fractionated as described in the test are shown. Vertical axes are adjusted for each sample to give comparable heights and are arbitrary. O, dense cells; ●, processed cells; Δ, least dense cells.

FIGURE 2: Calibration of Mean Photon Counts vs. Bulk Cellular ATP.

Unfractionated erythrocytes were treated with iodoacetamide and incubated at 37°. Aliquots were removed at intervals and chilled to 4° to stop ATP loss. Bulk samples were assayed by a 3-phosphoglycerate-coupled ATP assay (Sigma Chem. Corp. #366-UV), and single cell counts were obtained by laser lysis as described (2).

Fig 1

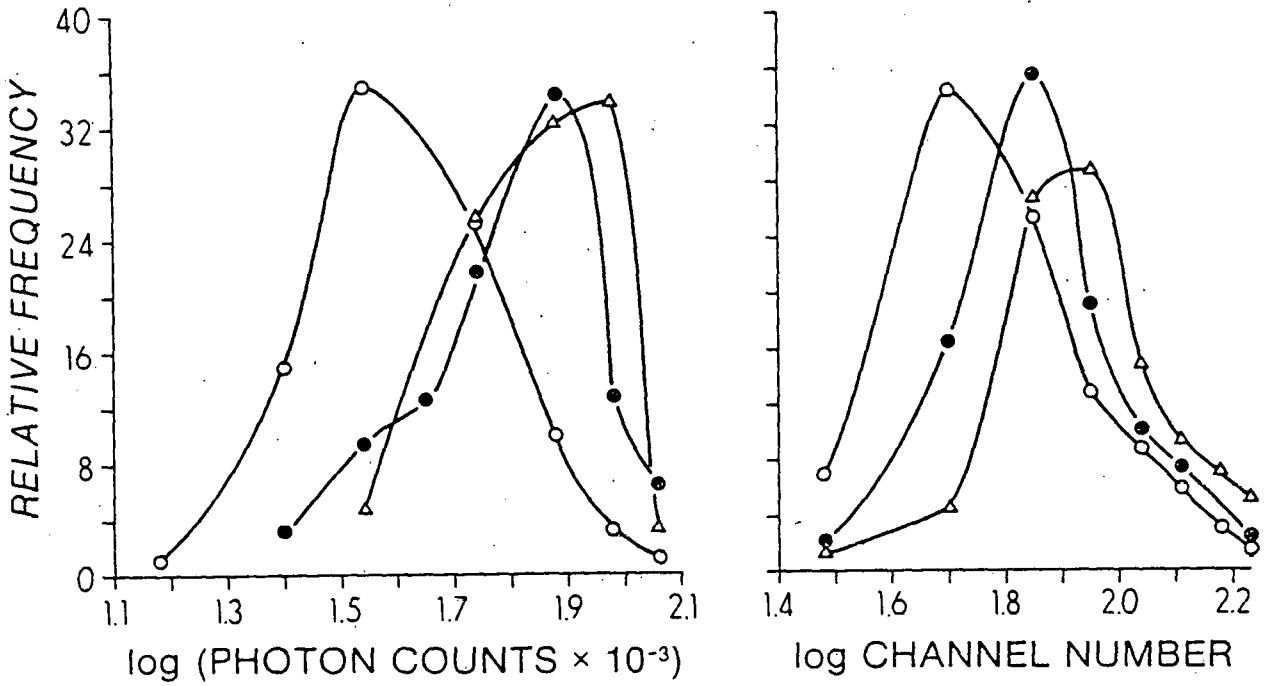
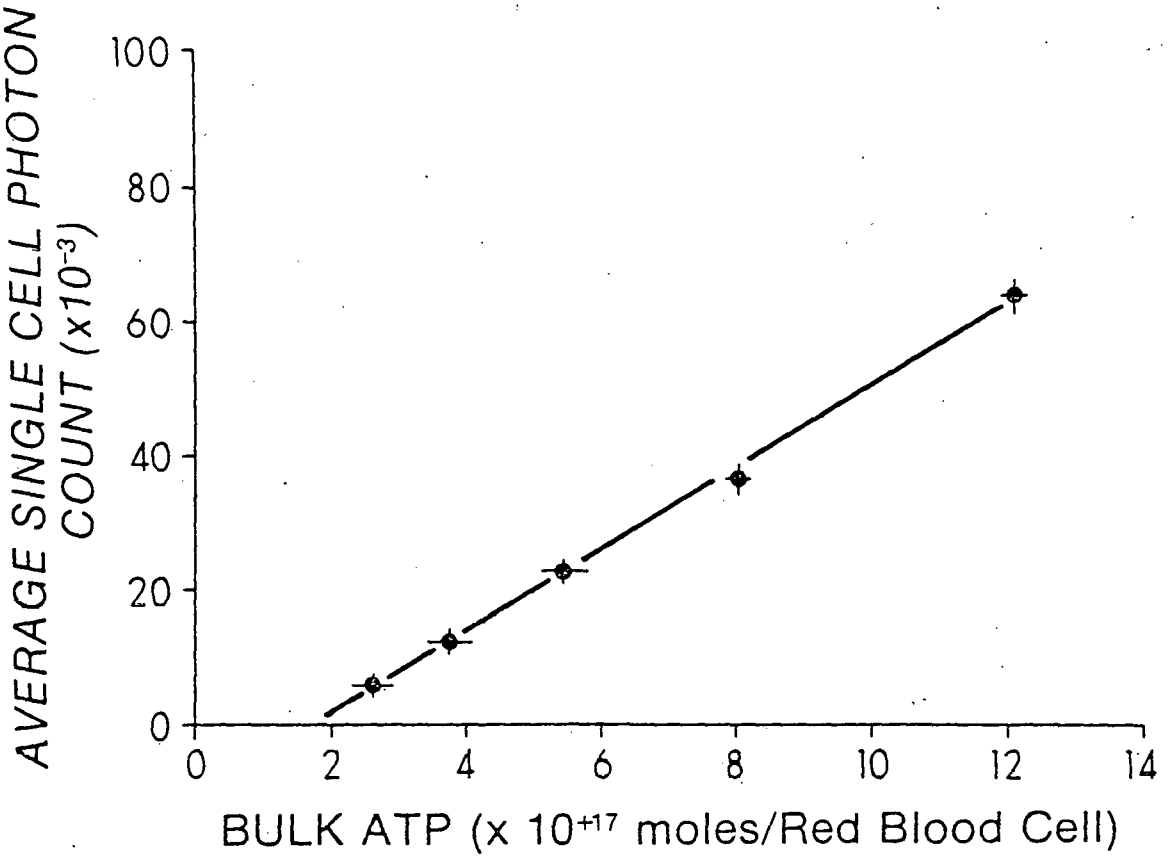

RELATIVE FREQUENCY
40
32
24
16
8
0
1.1
1.3
1.5
1.7
1.9
2.1
log (PHOTON COUNTS × 10⁻³)
1.4
1.6
1.8
2.0
2.2
log CHANNEL NUMBER

Fig 2


AVERAGE SINGLE CELL PHOTON COUNT (x10⁻³)
100
80
60
40
20
0
0
2
4
6
8
10
12
14
BULK ATP (x 10⁺¹⁷ moles/Red Blood Cell)