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The Decline of Catalytic Enzyme Activity Concentration of In Vivo Ageing Erythrocytes of the Man, the Dog and the Rat

Approach to a Quantitative Diagnostic Enzymology, IV. Communication

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In memoriam Professor Dr. Dr. Ivar Trautschold († 31. 01. 1984)

Summary: Human, dog and rat erythrocytes were separated by centrifugation on a discontinuous buffered Percoll gradient into fractions of progressively increasing mean cell age to measure the in vivo decline in catalytic activity of eleven enzymes during the erythrocyte lifespan. Erythrocyte enzymes decline exponentially at different rates and also differ between the species. The maximal and minimal catalytic activities (erythrocyte catalytic activity at the beginning and at the end of the appropriate erythrocyte life-span for a given species) and the intracellular half-life of enzymes were estimated. To test the hypothesis that circulating erythrocytes make a significant contribution to the normal catalytic activity in plasma it was assumed as a working hypothesis that the measured loss of catalytic activity in ageing erythrocytes is equivalent to the amount of the enzymes released in catalytically active form into plasma. This contribution was calculated.

Die Abnahme der katalytischen Enzymaktivitätskonzentration von in vivo alternden Erythrocyten bei Mensch, Hund und Ratte
Versuch der Begründung einer quantitativen Diagnostischen Enzymologie, IV.

Zusammenfassung: Erythrocyten von Mensch, Hund und Ratte wurden in einem diskontinuierlichen Percoll-Gradienten in Puffer in Fraktionen zunehmenden Alters separiert, um den in vivo-Abfall der katalytischen Aktivität von 11 Enzymen über die entsprechende Erythrocyten-Lebensdauer zu bestimmen. Die einzelnen Enzyme verlieren ihre katalytische Aktivität exponentiell mit unterschiedlicher Rate, auch unterschiedlich bei den untersuchten Spezies. Die maximalen und minimalen katalytischen Aktivitäten (katalytische Aktivität der Erythrocyten zu Beginn und zum Ende der entsprechenden Erythrocyten-Lebensdauer der untersuchten Spezies) und die intrazelluläre Halbwertszeit der Enzyme wurden bestimmt. Um die Hypothese zu testen, daß zirkulierende Erythrocyten einen wesentlichen Beitrag zur normalen katalytischen Enzymaktivität des Plasmas leisten, wird als Arbeitshypothese angenommen, daß der Verlust des alternden Erythrocyten an katalytischer Aktivität gleichbedeutend ist mit einer Freisetzung an katalytisch aktivem Enzym in das Plasma. Dieser Beitrag wird berechnet.

Introduction

For a long time it was assumed that the normal turnover of cells in the blood stream, especially of erythrocytes, made a significant contribution to the normal catalytic enzyme activity in plasma (1–4). Until now, however, this contribution has not been quantified and qualitative and theoretical considerations only have been considered. The erythrocyte loses its capability for protein synthesis shortly after entering the circulation. Throughout its life-span the

cell remains dependent on preformed enzymatic machinery (5). By exploiting different age-related alterations of erythrocytes, their fractionation according to age is possible (survey: l. c. (6)).

Over the years, density gradient centrifugation utilizing many different substances has become the most commonly used technique for fractionating erythrocytes of different mean age (7, 8).

This technique is based on the fact that during ageing in vivo the erythrocyte density increases (6, 8).

With respect to catalytic enzyme activities an interesting observation was made during these investigations. This observation was that there is a more or less pronounced in-vivo decline in intracellular catalytic activity with increasing age (6, 8, 9).

These latter results were obtained by isopycnic centrifugation through gradients of Percoll, a commercially available polyvinylpyrrolidone coated colloidal silica gel. This gel possesses certain advantages when compared to formerly used gradient material (6–8, 10, 11).

In these age related studies, the fixed absolute specific gravity of each cell layer and its catalytic activity was universally used as the density related parameter. Such a system fails to take into account that even though the variance of the erythrocyte distribution is constant for an individual, the mean erythrocyte specific gravity varies among individuals. *Piomelli* and coworkers (5, 12) proposed an alternative procedure. Based on a set of reasonable assumptions it is possible to establish a linear regression of the logarithm of catalytic activities in a fraction as a function of the probit of the density of the fraction. Using a modification of this method we have for the first time quantified the loss of catalytic activity for several enzymes of in-vivo ageing erythrocytes.

The data analysis was applied to the results of red cell separation according to age in a discontinuous Percoll gradient; the catalytic activity was determined in the erythrocyte layers of increasing age in man, the dog and the rat.

To obtain pure erythrocytes from blood, we developed a two-step discontinuous Percoll gradient. This gradient yields not only pure erythrocytes but pure polymorphonuclear granulocytes as well and may prove useful in this regard in the future.

Materials and Methods

Man and animals; blood drawing

Man

From male blood donors, aged from 26 to 37 years, 62–72 kg body weight, blood was drawn by venipuncture; n = 19

Dog

From male, unanaesthetized German shepherd dogs, weighing from 23 to 33 kg, blood was drawn from the cephalic vein; n = 22

Rat

Male rats from the inbred strain Lewis/Ztm, weighing 265–305 g and aged 100–120 days were used. Blood was withdrawn by catheter technique (13). Interestingly blood from heart puncture did not fractionate well and was therefore not suitable; n = 13

Blood was taken for all species between 08.00 and 09.00. Heparin was used as anticoagulant (man: 20 U/ml blood; animals: 50 U/ml blood). Samples were analysed immediately by the following fractionation and analytical methods.

Isolation of pure erythrocytes from blood by a two-step Percoll gradient

Solutions

NaCl 1.5 mol/l
NaCl 0.15 mol/l
Stock isoosmotic Percoll with NaCl: 9 vol Percoll (Pharmacia, Freiburg) from the bottle was mixed with 1 vol NaCl 1.5 mol/l. This Percoll solution was referred to as a 100% solution, and was then diluted to the subsequent desired concentration with NaCl 0.15 mol/l.

HEPES buffered isotonic saline with bovine serum albumin: NaCl 0.133 mol/l; KCl 0.0045 mol/l; bovine serum albumin (Cohn fraction V, Sigma) 35 g/l; HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid) 10 mmol/l, pH 7.3; 295 mosmol/kg H_2O, ρ = 1.012 kg/l.

Preparation of the two-step discontinuous gradient

Densities of the respective Percoll solutions for the different species, the blood volume added to the gradient, and the gravity forces applied to the gradient were as follow:

Man
76% (ρ = 1.0945 kg/l), 62% (ρ = 1.0779 kg/l), 4 ml blood, 350 *g* for 15 min.

Dog
76% (ρ = 1.0945 kg/l), 60% (ρ = 1.0756 kg/l), 3 ml blood mixed with 1 ml NaCl 0.15 mol/l, 350 *g* for 25 min.

Rat
81% (ρ = 1.1005 kg/l), 65% (ρ = 1.0815 kg/l), 3 ml blood mixed with 1 ml NaCl 0.15 mol/l, 350 *g* for 40 min.

Densities were calculated (14) and verified gravimetrically. Gradients were prepared in polystyrene centrifuge tubes (Sarstedt 250 No. 55.468, 13 ml, 95 × 16 mm) by underlayering 4 ml of the lighter layer of Percoll with 4 ml of the heavier layer by means of a syringe with a 22 G spinal-needle (0.7 × 88 mm, VYGON, Aachen). Blood was then applied to the top of the gradient and the tube was centrifuged at 20 °C in a Minifuge GL (Heraeus Christ, Osterode) centrifuge with a swing-out rotor.

Thrombocytes and mononuclear cells banded on the surface of the upper layer; polymorphonuclear cells banded at the interface of the two density concentrations, and erythrocytes banded at the bottom. These cells were removed by suction. This two-step gradient originally was developed for obtaining pure polymorphonuclear granulocytes in man (15). We modified this procedure with respect to the density concentrations of interest and adapted the method for the dog and rat. Erythrocytes from the bottom of the tube were washed twice with HEPES-buffered isotonic saline with bovine serum albumin;

The first washing was at 800 *g* for 10 min in the Minifuge, and the second in an Eppendorf centrifuge at 12000 *g* for 2 min. Different volumes of erythrocytes adjusted to a fraction of about 50% in HEPES buffered isotonic saline with bovine serum albumin were layered on top of a multi-step gradient for age differentiation.

Multi-step Percoll gradient for separation of red cells according to age

Solutions

HEPES-buffered isotonic saline with bovine serum albumin: see above.

HEPES-buffered stock solution: NaCl 1.33 mol/l, KCl 0.045 mol/l; HEPES 100 mmol/l; pH 7.3. Stock isotonic Percoll with HEPES and bovine serum albumin: 9 vol Percoll from the bottle were mixed with 1 vol HEPES-buffered stock solution. 35 g/l of bovine serum albumin were added. This Percoll solution was referred to as a 100% solution, which was then diluted to the respective desired concentrations with HEPES-buffered isotonic saline and bovine serum albumin; 300–310 mosmol/kg H_2O.

Preparation of multi-step discontinuous gradients

The following Percoll solutions were used for age differentiation of erythrocytes for the respective species:

Man
64%, 68%, 70%, 72%, 74%, 76%, 78% (ρ = 1.087–1.104 kg/l)

Dog
64%, 67%, 70%, 73%, 76%, 79% (ρ = 1.080–1.097 kg/l)

Rat
70%, 72%, 74%, 76%, 78%, 80%, 82% (ρ = 1.089–1.104 kg/l)

The relative densities were determined gravimetrically at 20 °C. Density differences of 2% between solutions (for man, rat) and 3% between solutions (dog) represented differences in mass concentrations of about 0.003 kg/l and 0.004 kg/l, respectively. It is in principle possible to produce discontinuous gradients with solutions different in density by as little as 0.002 kg/l (14).

The method described was modified from *Salvo* et al. (8) for the preparation of different density solutions. Density solutions for each set of species experiments were prepared in one batch, and were stored frozen at −20 °C because of limited stability at 4 °C (6). Gradients were prepared by superimposing 2 ml of each Percoll concentration into a polystyrene centrifuge tube using a multipurpose peristaltic pump (10) with a velocity of 8 ml/h.

Different volumes of washed erythrocytes separated by the two-step gradient and adjusted to a fraction of about 50% with HEPES-buffered isotonic saline with bovine serum albumin were applied to the top of the gradient and centrifuged in the Minifuge GL at 20 °C:

Man
1 ml erythrocyte suspension; 1000 *g*; 10 min

Dog
0.5 ml erythrocyte suspension; 1100 *g*; 20 min

Rat
0.5 ml erythrocyte suspension, 1300 *g*; 10 min.

The different gravity forces and times of suspension relate to different erythrocyte volumes for different species (16–18).

Cell layers were banded at the surface, the interfaces and the bottom. The very thin erythrocyte layers at the top and the bottom were excluded from catalytic activity determination. The least dense Percoll solution with lowest density was used for capturing the occasional (about 2‰) contamination with polymorphonuclear granulocytes. Considering the up to 400-fold higher catalytic activities of certain enzymes in polymorphonuclear cells (e. g. lactate dehydrogenase and phosphohexose isomerase) as compared to erythrocytes (10, 11), inclusion of this first layer could lead to erroneous results. Also both the uppermost and the lowest erythrocyte layers comprise fractions which stretch theoretically to infinity in each direction and are therefore not clear-cut and well defined populations.

The cell layers were collected from the gradient by aspiration with a peristaltic pump and washed twice with HEPES buffered isotonic saline with bovine serum albumin.

Each fraction was brought to a volume of 0.5 ml with HEPES-buffered isotonic saline with bovine serum albumin.

Reticulocytes

Reticulocytes counts were performed by counting 1000 cells stained with methylene blue.

Cell lysis

Cells were lysed by the addition of 0.5 ml ice-cold saturated digitonin solution containing the detergent ethylene polyethylene glycol, NP 40 (Fluka, Neu Ulm) to give final sample concentrations of 6.1 mmol/l and 5 ml/l for digitonin and NP 40, respectively. After standing for 30 min at 4 °C, the lysates were assayed for haemoglobin. They were not re-centrifuged prior to assay.

Haemoglobin

Haemoglobin was determined as cyanmethaemoglobin (19).

Determination of catalytic enzyme activity concentration

The different fractions were diluted to give a final sample of haemoglobin of about 0.1 g/l, which then was assayed for catalytic activity and was, if neccessary, further diluted with HEPES buffered saline with bovine serum albumin.

The catalytic activities of the following enzymes were determined:
lactate dehydrogenase (EC 1.1.1.27),
malate dehydrogenase (EC 1.1.1.37),
glucose-6-phosphate dehydrogenase (EC 1.1.1.49),
aspartate aminotransferase (EC 2.6.1.1),
hexokinase (EC 2.7.1.1),
pyruvate kinase (EC 2.7.1.40),
creatine kinase (EC 2.7.3.2),
adenylate kinase (EC 2.7.4.3),
acetylcholinesterase (EC 3.1.1.7),
aldolase (EC 4.1.2.13),
phosphohexose isomerase (EC 5.3.1.9).

In addition to the methods described in a previous publication (20), the following methods were applied for the following enzymes:
Glucose-6-phosphate dehydrogenase according to *Löhr & Waller* (21) (Boehringer, Mannheim).

Hexokinase according to *Seaman* et al. (5) in man and according to *Beutler* et al. (22) in other species (self-prepared).

Pyruvate kinase according to *Seaman* et al. (5) in man and according to *Beutler* et al. (22) in other species (self-prepared).

Acetylcholinesterase according to *Ellman* et al. (23) (Boehringer, Mannheim).

Hexokinase and pyruvate kinase were assayed at 37 °C and the activities were corrected to 25 °C with factors of 0.477 and 0.432, respectively (22).

Catalytic enzyme activities were expressed as U/g haemoglobin. All clinical chemical analyses were performed on the day of blood drawing.

Data analysis

The theoretical basis of the data analysis has been presented in detail by *Piomelli*'s group (5, 12), and has been used several times and verified by others (24–29). Our study is a more recent use of this technique. Essential modifications of his original procedure, related to our alternative quantitative approach to the catalytic activity decrease in ageing erythrocytes, are outlined in the discussion section. Briefly, we proceeded on the following assumptions:

1. The density of red cells increases as the cells age during their blood circulation (survey: l. c. (30)).

2. When normal erythrocytes are separated according to specific gravity a normal distribution is obtained (6, 31–33).

3. The mean specific gravity, although constant for each individual, varies from individual to individual. The variance of the distribution is always very similar (6, 8, 32, 33).

4. When several individuals are compared, the cumulative distribution function, rather than the absolute specific gravity, must be used for pooling of data.

5. The age of each erythrocyte fraction in our discontinuous gradient is determined by the position in the gradient.

6. As the mean cell haemoglobin (MCH) of the erythrocyte is a constant throughout the erythrocyte life-span (6, 8, 12, 34), a measured mass of haemoglobin should be directly proportional to the number of cells present.

7. The probit of the percentile position of each cell fraction in the gradient by haemoglobin determination can therefore be used as an indicator of cell age.

The normalized distribution of erythrocytes with respect to age is given by:

$$f(t) = \frac{1}{\sqrt{2\pi}} e^{-\frac{1}{2}t^2} \qquad \text{(Eq. 1)}$$

t = normalized erythrocyte age

The decline of intracellular enzyme catalytic activity in the age-related fractions of an erythrocyte population is approximately given by:

$$E(t) = E\mu \cdot e^{-\alpha t} \qquad \text{(Eq. 2)}$$

$E\mu$ = catalytic activity per g haemoglobin at mean age
α = velocity constant of catalytic activity decline

The differential change of catalytic activity per g haemoglobin in a single infinitesimal age fraction or time interval is given by:

$$-\frac{dEi}{dt} = \frac{dEe}{dt} = E\mu\alpha e^{-dt} \qquad \text{(Eq. 3)}$$

Ei; Ee = intracellular and extracellular catalytic activity per g haemoglobin.

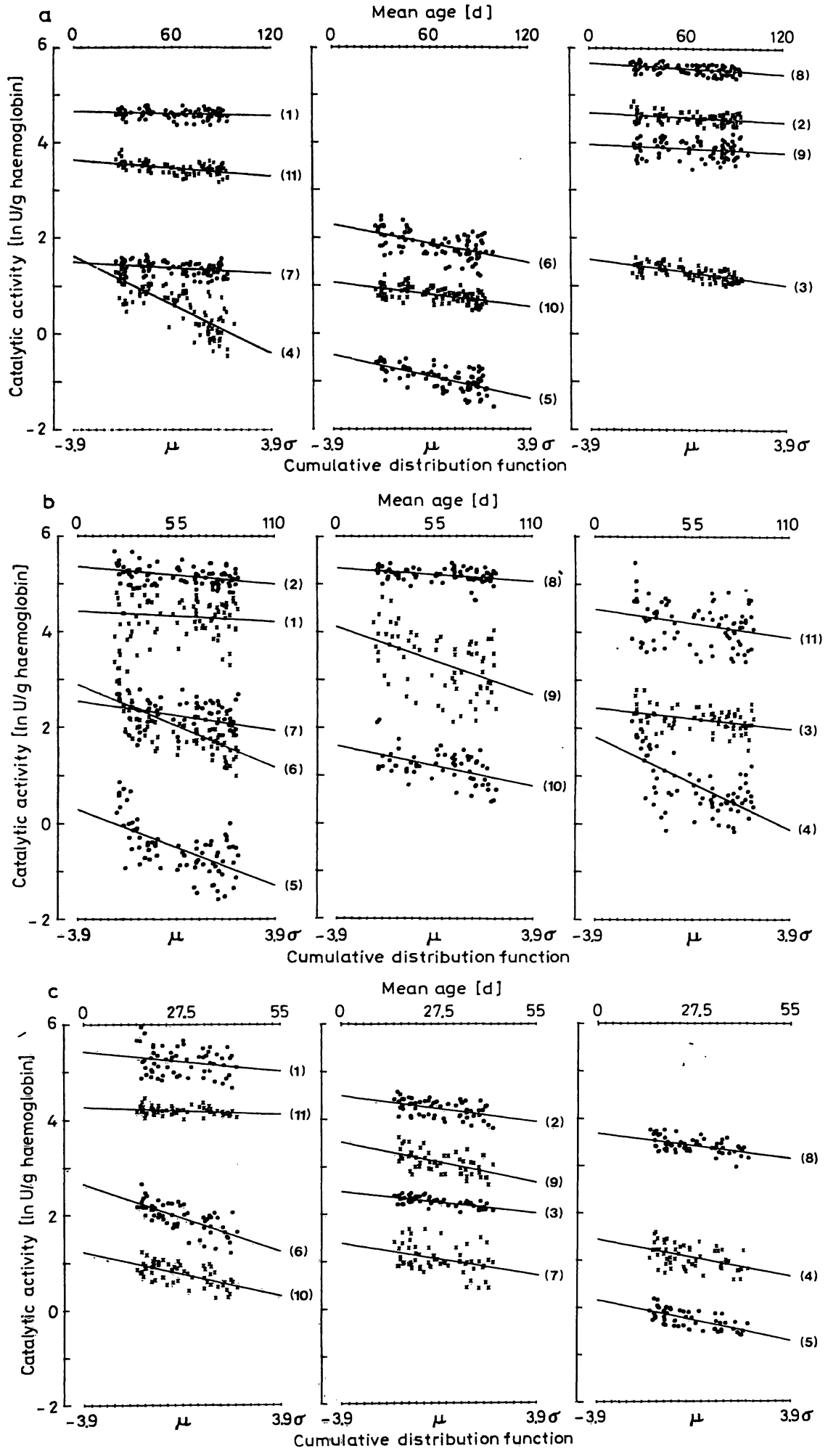
As the erythrocytes are normally distributed in relation to age, each dE/dt must be multiplied by the respective differential frequency and the resulting products must be integrated to sum the differential catalytic activity of all erythrocytes per g haemoglobin and infinitesimal time interval:

$$\frac{dEe\,total}{dt} = \int_{-\infty}^{\infty} \left(\frac{1}{\sqrt{2\pi}} e^{-\frac{1}{2}t^2} \cdot E\mu\alpha e^{-\alpha t} \right) dt \qquad \text{(Eq. 4)}$$

$$\frac{dEe\,total}{dt} = E\mu\alpha e^{\frac{1}{2}\alpha^2} \qquad \text{(Eq. 5)}$$

The in-vivo life-span of the age-dependent enzymes was estimated from the slope of the regression line for age vs. activity (fig. 1 a–1 c).

Fig. 1 a, b, c. Rate of decline of erythrocyte catalytic activities in
a) human,
b) dog and
c) rat erythrocytes
with age.
The slope of the regression line is obtained by least squares regression of the logarithm of catalytic activity vs. the probit transformation of the midpoint cumulative percentile of the age-separated fractions.
Number in parentheses refer to the following enzymes:
(1) lactate dehydrogenase,
(2) malate dehydrogenase,
(3) glucose-6-phosphate dehydrogenase,
(4) aspartate aminotransferase,
(5) hexokinase,
(6) pyruvate kinase,
(7) creatine kinase,
(8) adenylate kinase,
(9) acetylcholinesterase,
(10) aldolase,
(11) phosphohexose isomerase.


a
Mean age [d]
0 60 120
Catalytic activity [ln U/g haemoglobin]
6 4 2 0 -2
(1)
(11)
(7)
(4)
(6)
(10)
(5)
(8)
(2)
(9)
(3)
-3.9 μ 3.9σ
Cumulative distribution function
b
Mean age [d]
0 55 110
(2)
(1)
(7)
(6)
(5)
(8)
(9)
(10)
(11)
(3)
(4)
c
Mean age [d]
0 27.5 55
(1)
(11)
(6)
(10)
(2)
(9)
(3)
(7)
(8)
(4)
(5)

The levels in the gradient, corresponding to cells of ages 0 and the last day of the respective live-span for a given species, were taken as a distance of + 3.9 standard deviations from the midpoint. This distance corresponds to the point where the extrapolated slope of decline in the gradient for reticulocytes (age = 0 days) intercepts the 100% level. As the gradient is symmetrical, the level at which a fraction containing 100% cells of maximal possible age should be an equal distance from the midpoint. Our experiments, however, like others before it, measured only cells located in a distance of only about + 2.5 sigma (5, 6, 24–29), and extrapolation to + 3.9 sigma is nesseccary to obtain maximal (day 0) and minimal (end of life-span) catalytic activities. To verify the borderline obtained from reticulocyte count we additionally chose an alternative approach. We established a continuous linear density gradient (using the same solutions as for the discontinuous gradients) so as to stretch the left (i. e. light) tail of the erythrocyte distribution over a wide range of 11 ml of continuously formed gradient solution. This left or light tail was separated into 50 fractions of 200 µl each and haemoglobin was determined by reducing the original 250 : 1 dilution with *Drabkin*'s solution (19) to a ratio of 10 : 1. This procedure allows for direct measurement of a range to −3.8 sigma. Results obtained in this way agreed well with results obtained by extrapolation to −3.9 sigma from the narrower experimental range data. This verifies the values obtained from reticulocyte extrapolation.

Results

The mean recovery ($\bar{x} \pm$ standard deviation) of cells in the multi-step gradient expressed as fraction of total cells applied to the gradient was:

Man: 98.8 ± 6.3%
Dog: 101 ± 5.1%
Rat: 103 ± 9.7%

The distribution of cells along the gradient as compared to a normal distribution yields the following correlation coefficients ($\bar{x} \pm$ SD):

Man: 0.985 ± 0.008
Dog: 0.987 ± 0.007
Rat: 0.984 ± 0.017

Figures 1a, b and c show the rate of decline of catalytic enzyme activities with erythrocyte ageing in man, dogs and rats.

The correlation coefficients of the resulting regression lines were significant (2p at least < 0.05) except for acetylcholinesterase in man and for lactate dehydrogenase in all species.

From the slope of the regression line a different decline of catalytic activities was seen. These are reported below in order of fastest to slowest rates of decline:

Man

Aspartate aminotransferase, hexokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase, aldolase, phosphohexose isomerase, creatine kinase, adenylate kinase, malate dehydrogenase, acetylcholinesterase, lactate dehydrogenase.

Dog

Aspartate aminotransferase, pyruvate kinase, hexokinase, acetylcholinesterase, aldolase, creatine kinase, phosphohexose isomerase, glucose-6-phosphate dehydrogenase, malate dehydrogenase, adenylate kinase, lactate dehydrogenase.

Rat

Pyruvate kinase, aldolase, hexokinase, acetylcholinesterase, aspartate aminotransferase, creatine kinase, malate dehydrogenase, adenylate kinase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase, phosphohexose isomerase.

Based on equation 5 of our data analysis the daily loss of catalytic activity of total body erythrocytes in man, dogs and rats is given in table 1 in units (U) lost per 24 h. The following data were used for calculation for the respective species:

Man

Mean body weight of probands: 67 kg; blood volume: 71 ml/kg body weight (35); total blood volume: 4.8 l; blood haemoglobin concentration: 151 g/l; total body haemoglobin content: 725 g; erythrocyte life-span: 120 days.

Tab. 1. Daily loss of catalytic activity (U/24 h) of total body erythrocytes in man, dogs and rats calculated from the respective regression lines of fig. 1a, 1b, 1c and equation 5 of the data analysis.

	Man	Dog	Rat
Lactate dehydrogenase	49.1	60.3	3.40
Malate dehydrogenase	98.8	255	1.67
Glucose-6-phosphate dehydrogenase	11.6	15.9	0.20
Aspartate aminotransferase	22.9	18.5	0.10
Hexokinase	2.21	3.98	0.03
Pyruvate kinase	31.4	54.4	0.46
Creatine kinase	5.63	23.3	0.09
Adenylate kinase	356	213	0.74
Acetylcholinesterase	45.1	174	0.87
Aldolase	6.91	11.2	0.09
Phosphohexose isomerase	65.3	155	0.44

The following data were used for calculation:

Man: Mean body weight of probands: 67 kg; blood volume: 71 ml/kg (35); total blood volume: 4.8 l; blood haemoglobin concentration: 151 g/l; total body haemoglobin content: 725 g; erythrocyte lifespan: 120 days.

Dog: Mean body weight: 27.9 kg; blood volume: 100 ml/kg (36); total blood volume: 2.79 l; blood haemoglobin concentration: 158 g/l; total body haemoglobin content: 441 g; erythrocyte lifespan: 110 days (37).

Rat: Mean body weight: 290 g; blood volume: 61 ml/kg (38); total blood volume: 17.7 ml; blood haemoglobin concentration: 139.5 g/l; total body haemoglobin content: 2.5 g; erythrocyte lifespan: 55 days (37, 39).

Dog

Mean body weight of dogs: 27.9 kg; blood volume: 100 ml/kg body weight (36); total blood volume: 2.79 l; blood haemoglobin concentration: 158 g/l; total body haemoglobin content: 441 g; erythrocyte lifespan: 110 days (37).

Rat

Mean body weight of rats: 290 g; blood volume: 61 ml/kg body weight (38); total blood volume: 17.7 ml; blood haemoglobin concentration: 139.5 g/l; total body haemoglobin content: 2.5 g; erythrocyte lifespan: 55 days (37, 39).

Tables 2a, b and c summarize the catalytic activites of enzymes at various stages and the intracellular half-lives of enzymes in man, dogs and rats. In the case of almost every enzyme studied, the half-life exceeded the lifespan of the erythrocyte itself. This finding indicates that the oldest circulating cells still retain more than half their maximal catalytic activity. Only a few enzymes had a half-life shorter than the appropriate erythrocyte lifespan itself.

Tab. 2a. Catalytic activities (U/g Hb) at various times and intracellular half-lives (t/2 in days) of erythrocyte enzymes in man. t = 0, Eμ, t = 120: catalytic activities at age 0, 60 and 120 days; $\bar{x} \pm SD$ = catalytic activities of unfractionated erythrocytes.

	t = 0	t = 120	Eμ	$\bar{x} \pm SD$	t/2
Lactate dehydrogenase	104	96.0	100	96.5 ± 18.8	1024
Malate dehydrogenase	102	85.7	93.5	88.3 ± 11.1	476
Glucose-6-phosphate dehydrogenase	4.75	2.80	3.65	3.34 ± 0.34	158
Aspartate aminotransferase	4.98	0.68	1.84	1.25 ± 0.27	41.7
Hexokinase	0.63	0.26	0.40	0.37 ± 0.07	91.7
Pyruvate kinase	9.64	4.33	6.46	5.42 ± 1.9	104
Creatine kinase	4.46	3.52	3.96	3.65 ± 0.45	354
Adenylate kinase	295	236	264	248 ± 30.0	372
Acetylcholinesterase	52.7	45.2	48.8	45.3 ± 13.9	544
Aldolase	2.89	1.74	2.25	2.07 ± 0.35	164
Phosphohexose isomerase	37.9	27.1	32.0	31.6 ± 4.8	247

Tab. 2b. Catalytic activities (U/g Hb) at various times and intracellular half-lives (t/2 in days) of erythrocyte enzymes in dogs. t = 0, Eμ, t = 110: catalytic activities at age 0, 55 and 110 days; $\bar{x} \pm SD$ = catalytic activities of unfractionated erythrocytes.

	t = 0	t = 110	Eμ	$\bar{x} \pm SD$	t/2
Lactate dehydrogenase	83.3	68.2	75.4	83.3 ± 19.9	382
Malate dehydrogenase	213	149	179	179 ± 43.7	214
Glucose-6-phosphate dehydrogenase	11.3	7.30	9.06	9.27 ± 1.65	175
Aspartate aminotransferase	6.09	0.88	2.32	1.99 ± 0.56	39.4
Hexokinase	1.35	0.27	0.61	0.66 ± 0.25	47.5
Pyruvate kinase	18.2	3.19	7.61	7.46 ± 2.75	43.8
Creatine kinase	12.8	6.95	9.44	9.67 ± 3.27	124
Adenylate kinase	210	157	181	185 ± 26.3	261
Acetylcholinesterase	61.2	14.7	30.0	29.8 ± 11.8	53.5
Aldolase	5.04	2.19	3.32	3.75 ± 0.70	91.1
Phosphohexose isomerase	88.5	49.3	66.1	67.8 ± 23.1	131

Tab. 2c. Catalytic activities (U/g Hb) at various times and intracellular half-lives (t/2 in days) of erythrocyte enzymes in rats. t = 0, Eμ, t = 55: catalytic activities at age 0, 27.5 and 55 days; $\bar{x} \pm SD$ = catalytic activities of unfractionated erythrocytes.

	t = 0	t = 55	Eμ	$\bar{x} \pm SD$	t/2
Lactate dehydrogenase	226	151	185	182 ± 40.8	94.2
Malate dehydrogenase	89.0	52.0	68.0	75.7 ± 18.4	70.9
Glucose-6-phosphate dehydrogenase	12.0	7.54	9.50	9.42 ± 2.1	82.7
Aspartate aminotransferase	4.24	1.91	2.84	3.30 ± 0.73	47.9
Hexokinase	1.18	0.48	0.76	0.77 ± 0.13	42.5
Pyruvate kinase	14.2	3.44	6.99	7.59 ± 1.8	26.9
Creatine kinase	4.03	2.03	2.86	2.79 ± 0.68	55.4
Adenylate kinase	39.9	23.4	30.6	33.2 ± 6.8	71.7
Acetylcholinesterase	34.1	14.5	21.8	21.9 ± 5.6	44.6
Aldolase	3.41	1.36	2.15	2.38 ± 0.45	41.4
Phosphohexose isomerase	71.1	61.3	66.0	69.9 ± 9.9	257

There was good agreement between the values for unfractionated erythrocytes and erythrocytes of mean age. This demonstrates that the fractionation procedure itself does not alter enzyme activity.

Discussion

The advantageous properties of Percoll, such as its low viscosity, low osmotic pressure, non-toxicity and its inability to penetrate cell membranes have resulted in its wide replacement of formerly used gradient material for separating various cell types (8, 10, 11, 14). The employment of a discontinuous Percoll gradient for red cell fractionation with the closest approximation to physiological conditions by using buffered Percoll containing physiological levels of sodium, potassium and albumin was first introduced by *Rennie* et al. (6). Our procedure essentially follows *Salvo*'s (8) modifications, with the following additional changes:

1. Easier steps in preparing Percoll solutions of different density.

2. Red cell separation according to age in buffered Percoll was adapted for the first time to dogs and rats.

3. Red cells were obtained free of leukocytes and platelets by centrifugation of whole blood on a two-step discontinuous gradient as modified from *Hjorth* et al. (15) for man and adapted to dog and rat, thus continuously maintaining more physiological conditions for red blood cells than during filtration of blood through microcrystalline cellulose (6, 8, 22).

4. Our multistep gradient consisted of six (dog) or seven (man, rat) different densities of Percoll solutions instead of four (8), resulting in higher resolution of total red blood cell population.

5. Cell lysis was performed by the addition of detergent to ensure distribution of nearly all catalytic activity of membrane-integrated or membrane-associated enzyme in the soluble fraction (10, 11).

Our data on red cell catalytic activity decline during ageing strongly confirm previous observations in various species employing different gradient material (5, 9, 12, 40–46).

Previous studies using Percoll have measured only a few enzymes in a small number of cases (6, 8). In every case the fixed absolute specific gravity of each cell layer and its catalytic activity was used. Nevertheless, the following detailed observations of our study are in agreement with these previous findings:

1. There is a differential decline of enzyme catalytic activities in man with ageing (aspartate aminotransferase > pyruvate kinase > hexokinase > glucose-6-phosphate dehydrogenase > aldolase > lactate dehydrogenase) (5, 6, 8, 9).

2. The total erythrocyte population is normally distributed (6, 32, 33).

3. Catalytic activities of Percoll-fractionated total erythrocyte population in man, dogs and rats (tab. 2 a, b, c) were sometimes several-fold higher than previously reported and confirms values reported by us for the main blood cell classes among erythrocytes of man and animals (10, 11).

Although absolute values for many enzymes differed from previous reported data, the rates of decline agreed well with one another. Our modifications of *Piomelli*'s original data analysis (5, 12) are related to our alternative approach.

He wanted to determine the decline from maximal catalytic activity of reticulocytes (day 0 cells) to minimal catalytic activity (day 120 cells), a time course which experimentally cannot be completely measured from start to finish. By extrapolation, however, from the experimentally accessible decline between distances of about $\pm$ 2.5 sigma from the midpoint to a distance of $\pm$ 3.9 sigma, these values for maximal and minimal activity can be reasonably obtained. The validity of this approach was confirmed by our alternative procedure of haemoglobin determination of the left-tailed erythrocyte distribution curve from a linear continuous gradient. We additionally wanted to obtain quantitative data on the loss of catalytic activity, which based on our working hypothesis, is released from erythrocytes in a catalytically active form. Briefly, we were more interested in what was lost than in what was left. This approach, however, had to take into account the frequency distribution pattern.

A distance of $\pm$ 2.5 sigma, comprising 98.6% of the total distribution, was chosen. A sufficient amount of the total distribution curve could then, in fact, be directly measured experimentally. This can be seen in figure 1 a, b, c.

As outlined in the methods and material sections, values exceeding these borders were excluded from catalytic activity determination as they involve the risk of contamination with leukocytes (at the top of the gradient) or represent an infinitely dense population (both at the top and the bottom).

Circulating normal red blood cells are ultimately sequestered within the confines of the mononuclear phagocyte system. A variety of mechanisms to explain how aged red cells are recognized have been proposed. These include metabolic deficiency related to decay of enzyme catalytic activity (47), immune elimination (48, 49), membrane oxidation (50–53) and alteration of cell shape, surface and rheological properties (50, 54–57).

The most studied and striking event during the in-vivo ageing of mammalian erythrocytes is the apparent decline of the cellular catalytic activity of numerous enzymes (for surveys up to 1982: l. c. (8, 47)).

It is not evident from the data available whether this loss represents an inactivation of the enzyme with age or a release of enzyme in an active form. Very few enzymes have been investigated to date with respect to the etiologic basis of enzyme catalytic activity decline. For glucose-6-phosphate dehydrogenase, hexokinase, aldolase, aspartate aminotransferase and superoxide dismutase, changes in kinetic properties (42, 43, 58–60), thermostability (42, 58), the isoelectric point (59) and/or electrophoretic mobility (61) point towards kinetic, isozymic and structural modifications of these enzymes. The formation of a "cross-reactive material", i. e. protein reacting with specific antibodies but devoid of enzymatic activity, has been shown for superoxide dismutase and aldolase (40, 52, 53, 60).

No alterations in electrophoretic mobility, however, were observed for glucose-6-phosphate dehydrogenase and lactate dehydrogenase (58, 61). In reviewing possible causes of inactivation of red cell enzymes *Bartosz* (44) concluded that "all the systems used failed to reproduce the sequence of enzyme inactivation observed during in-vivo ageing of the erythrocyte". The question of whether catalytic activity is lost to some extent by release of catalytically active enzymes by ageing erythrocytes, however, remains. Evidence supporting this hypothesis comes from in-vitro experiments where erythrocytes were incubated in *Krebs-Ringer* buffer or autologous plasma. For example, release of lactate dehydrogenase calculated by determination in supernatant and/or in haemolysate amounts to 0.6% per 24 h (62, 63).

This compares with a value of 0.07% found in our study for in-vivo ageing of erythrocytes.

The only attempt, prior to our study, to explain experimentally the origin of cell enzymes in normal blood plasma was undertaken by *Friedel & Mattenheimer* (4).

In a comparative study they investigated which of 10 major organs, including blood cells, could be excluded as significant sources of lactate dehydrogenase in normal plasma. This was based on an evaluation of the different isoenzyme patterns in plasma and tissue. They concluded that the major portion of lactate dehydrogenase in normal plasma derives from erythrocytes and thrombocytes. The same was also said to be true for all other enzymes found in erythrocytes and thrombocytes. Some of their basic assumptions and findings, however, are open to question:

1. They establish an isoenzyme pattern in rat plasma and erythrocytes of exclusively isoenzyme 5 (M 4). The determination of α-hydroxybutyrate dehydrogenase activity, however, which is selective for isoenzyme 1 (H 4) rather than specific (64), yielded values for plasma and erythrocytes of 40% (65) and 25% (*Lindena*, unpublished) of the total lactate dehydrogenase catalytic activity.

2. Based on an assumption of nearly equal catalytic activities for lactate dehydrogenase and creatine kinase in striated muscle, and the finding of a 10-fold higher lactate dehydrogenase activity in plasma as compared to creatine kinase activity, they concluded that only a small portion of plasma lactate dehydrogenase catalytic activity is muscle derived. The assumption of nearly equal catalytic activities for lactate dehydrogenase and creatine kinase in muscle, however, was based on measurement of creatine kinase with an unreliable method (measurement of unactivated enzyme with the so-called "forward" reaction).

We know today that both enzymes are found in plasma in nearly the same order of magnitude, whereas in skeletal muscle the catalytic activity of creatine kinase is 5–10-fold higher than for lactate dehydrogenase (20, 66).

Normal creatine kinase catalytic activity in plasma originates mainly from muscle. It follows that other enzymes found in quantity in muscle (e. g. lactate dehydrogenase) should also contribute significantly to plasma enzyme catalytic activity, especially when one considers that 40% of total body mass is made up of striated muscle. Similar arguments hold true

for contributions of the heart, liver and other organs to plasma catalytic activity.

In the present study we undertook to quantify the in-vivo loss of catalytic activity of the ageing erythrocyte. The extent to which, if at all, ageing erythrocytes contribute to the apparent catalytic activity in plasma has not yet been definitely determined. Current work in our laboratory is underway to answer this question.

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