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AGE-DEPENDENT CHANGES IN VOLUME AND HAEMOGLOBIN CONTENT OF ERYTHROCYTES IN THE CARP (*CYPRINUS CARPIO* L.)

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Summary

Carp erythrocytes were fractionated by angle-head centrifugation which yielded fractions with a linear increase in density. Haematological examinations revealed that the heavier red blood cells of carp had greater volumes (MCV), more haemoglobin (MCH) and higher haemoglobin concentrations (MCHC) than light ones. The same experiments with human red cell fractions yielded a decrease in MCV, constant MCH and an increase in MCHC. Haemoglobin content in individual erythrocytes was also determined by scanning stage absorbance cytophotometry to establish the frequency distribution of the cellular haemoglobin contents. In carp, the distribution was symmetrical with the means increasing with density. No such change with cell density was found in human erythrocytes.

Both carp and human erythrocytes incorporated [2-^{14}C]glycine *in vitro*. After gel filtration, radioactivity was detected in carp, but not in human, haemoglobin fractions. ^{14}C was found in all three haemoglobin fractions, obtained by isoelectric focusing, and was present in the haem and in the globin. [2-^{14}C]glycine-labelled erythrocytes were reinjected into chronically cannulated carp and followed *in vivo* for several months. With time, the main peak of scintillation counts shifted from red cell fractions of low to high density.

This is considered as evidence that density and age of red cells in carp are positively correlated and that erythrocytes can synthesize haemoglobin while circulating in the peripheral blood.

Introduction

Carp (*Cyprinus carpio* L.) erythrocytes, like the red blood cells in all non-mammalian vertebrates, retain their nucleus and some cytoplasmic organelles while circulating in the peripheral blood (Yasuzumi & Higashizawa, 1955; Weinreb, 1963; Sekhon & Beams, 1969; Iuchi & Yamamoto, 1983). Whether

Key words: carp erythrocytes, carp haemoglobin, density separation, ^{14}C-labelling, cytophotometry.

these erythrocytes solely transport idle ballast at the expense of cell deformability and maximum load, and if they are still capable of synthesizing haemoglobin, are not yet known.

Fish erythrocytes appear in the peripheral blood at rather early stages of development (Haider, 1967; Yamamoto & Iuchi, 1976; Härdig, 1977; Kreutzmann & Jonas, 1978). Thus, normal fish blood usually contains a small proportion of immature red cells, easily identifiable from their circular rather than elliptical shape. In individuals that have previously suffered respiratory stress (i.e. hypoxia or bleeding), this population may be increased temporarily and with a latency of some days, whereas an increase in mature red cells in cyprinids seems to be unusual (Krzymowska *et al.* 1960; Zanjani *et al.* 1969; Chudzik & Houston, 1983; Schindler & de Vries, 1986). Intravascular differentiation into mature red cells involves changes in the shape, loss of various organelles, changes in the concentrations of bioactive substances, changes in physical properties and an accumulation of haemoglobin (Sekhon & Beams, 1969; Iuchi & Yamamoto, 1983; Lane & Tharp, 1980; Lane *et al.* 1982). Differentiation between mature and immature cells would be even more difficult if maturity were defined as the stage of terminated haemoglobin synthesis.

From the present paper it is clear that circulating carp erythrocytes continue to synthesize haemoglobin. This evidence is based on experiments using ^{14}C-labelled glycine *in vivo* and *in vitro* and separating the erythrocytes by density fractionation.

Materials and methods

Blood sampling

Genetically uniform carp (*Cyprinus carpio*, approx. 2000 g) were obtained from a state-controlled hatchery and acclimatized for at least 4 weeks in a large, well-aerated tank (3000 l) supplied with running tap water at 180 l h^{-1} at 14–16°C and with oxygen. Maintenance and surgical procedures for chronic cannulation of the dorsal aorta followed Hughes *et al.* (1983). Tricaine methane sulphonate (MS222, Sandoz; 0·1 g l^{-1}) served as anaesthetic during surgery. Blood was sampled from free-swimming specimens after a minimum of 3 days of recovery. Care was taken not to perturb the fish. To prevent coagulation, 150 i.u. of sodium heparin (Vetren, Promonta, FRG) was added to 5 ml of blood. With less heparin, clotting of carp blood was not completely inhibited and the erythrocytes tended to aggregate.

To compare carp erythrocytes with non-nucleated mammalian erythrocytes, all tests, except for *in vivo* labelling, were also performed with human blood obtained from healthy volunteers.

Density centrifugation

The plasma and buffy coat of 5 ml samples of blood were removed by centrifugation (Christ IV KS, Osterode, FRG) at 900 *g* and 4°C for 10 min. The erythrocytes were washed twice with either 0·9 % NaCl (human red cells) or

physiological carp saline (PCS), using the method of Imamura (1979), and modified to give a total osmolarity of 280 mosmol l^{-1} and pH 7·9: PCS contains (in mmol l^{-1}): NaCl, 141·1; KCl, 1·43; $CaCl_2$, 0·99; $NaHCO_3$, 2·64; glucose, 6·16. Washing, suspending and diluting procedures were always carried out in the appropriate saline. Resuspended erythrocytes, adjusted to a haematocrit of approximately 0·8, were transferred to special, long narrow polypropylene tubes (length 90 mm, diameter 4 mm, volume 0·9 ml) using capillary syringes. Centrifugations were performed for 40 min in a Sorvall RC 2B-SS 34 rotor (Du Pont, Wilmington, USA), either at 15 °C and 20 000 *g* for carp red cells or at 25 °C and 20 000 *g* for human red cells. The columns of red cells were each separated into five or six equal parts by means of glass capillaries stuck to plastic syringes. The syringes were filled with the resuspension medium to give a haematocrit of about 0·4. In some experiments specimens were used some weeks after bleeding. These samples showed a typical light red zone at the top which represented very young red cells. This light red zone was removed and treated as a fraction on its own.

Estimation of density, MCV, MCH and MCHC

After separation, densities of fractionated red cells and nonfractionated red cells of the whole blood were measured by centrifuging parts of the fractions in density gradients composed of dimethyl phthalate and dibutyl phthalate at a ratio resulting in 10 steps of specific gravity between 1·0869 and 1·1278 (Danon & Marikovsky, 1964).

The haematocrit (Hct) was determined by means of a Heraeus Christ microfuge HC 101 (Osterode, FRG). The haemoglobin concentration was determined by the haemoglobin–cyanide method (Betke & Savelsberg, 1950; van Kampen & Zijlstra, 1961) using an Eppendorf photometer 1101 M (Hamburg, FRG). The red cell count (RBC in 10^{-12} l) was obtained by Coulter counter model D (Harpenden, Herts, UK).

From these data the following variables were calculated: mean corpuscular volume (MCV) = Hct/RBC (in fl = 10^{-15} l); mean corpuscular haemoglobin content (MCH) = haemoglobin concentration/RBC (in pg red blood cell $^{-1}$); mean corpuscular haemoglobin concentration (MCHC) = haemoglobin concentration/Hct (in g l^{-1} red blood cells).

Scanning stage absorbance cytophotometry

The haemoglobin content of individual erythrocytes was measured by scanning stage absorbance cytophotometry (Schindler *et al.* 1985). For preparations used in microscope photometry, two drops of resuspended red cells from each fraction of either carp or human donors were fixed for 3 h in 5 ml of 0·5 % glutaraldehyde in isotonic salt solutions; for carp cells, 140·2 mmol l^{-1} NaCl, 4·0 mmol l^{-1} KCl, 1·5 mmol l^{-1} $MgCl_2 . 6H_2O$, 3·6 mmol l^{-1} $NaHCO_3$, 0·5 mmol l^{-1} $CaCl_2$, adjusted to pH 7·9; for human cells, Ringer's solution USP (NaCl, 147·5; KCl, 4·0 and $CaCl_2 . 2H_2O$, 2·25 mmol l^{-1}). To ensure complete oxidation to methaemoglobin, $NaNO_2$ was added to the fixatives to give a final concentration of 10 mmol l^{-1}.

Afterwards, the suspensions were centrifuged and the pellets resuspended in 20 ml of 0·5 mmol l^{-1} phosphate buffer (pH 7·5). Washing was repeated four times. The suspensions of fixed erythrocytes were stored at −80°C.

Preparations were made by spraying the thawed samples with an atomizer onto slides immediately followed by drying in a stream of compressed air. About 100 single cells of each fraction were measured and the results plotted as a frequency distribution. In scanning cytophotometry of individual cells, the integrated absorbance value (the sum of local absorbance values after subtraction of the mean background value) was recorded, together with the number of object points. The integrated absorbance of fixed cells at 411 nm (Soret band of methaemoglobin) represents physically the amount of haemoglobin within the cells and, for convenience, is given in arbitrary units (AU).

Isotope labelling and measurements of ^{14}C-radioactivity

For examination of the fate of [2-^{14}C]glycine-labelled erythrocytes *in vivo*, blood samples of 20 ml were taken from six carp. After removal of the plasma and washing twice, the red cells were incubated in PSC, the suspensions being adjusted to the original haematocrit of each specimen, supplemented with 20 μCi of [2-^{14}C]glycine (52 mmol l^{-1}, Amersham, Braunschweig, FRG) at 18°C for 10 h in a shaking water bath. Afterwards, the cells were washed three times, resuspended in the original plasma at 4°C and reinjected into the dorsal aorta.

In the course of 7 months, blood samples of about 5 ml were taken from each carp at irregular intervals. One of the six fishes died after 210 days. After density separation, radioactivity and cell counts were estimated in each fraction using a Tricarb liquid scintillation counter (Packard, Illinois, USA) and Coulter counter, respectively. Combustion in a Tricarb oxidizer preceded liquid scintillation counting. The radioactivity of each fraction was determined as counts per minute per 10^9 cells and expressed as a percentage of the total.

Red cells from human heparizined venous blood (10 ml) were washed and incubated *in vitro* with 5 μCi of [2-^{14}C]glycine in Ringer's solution enriched with 6 mmol l^{-1} glucose at 37°C for 10 h. The erythrocytes were washed three times, resuspended in the original plasma (supplemented with heparin, 30 i.u. ml^{-1}) and erythrocyte fractions were examined 2 days after labelling.

Gel filtration technique, IEF, splitting of haem from globin

Following isotope labelling, small samples of carp and human erythrocytes were diluted in 1 mmol l^{-1} Tris-HCl buffer pH 8·3 and pH 8·0, respectively. The cells were haemolysed by repeated quench-freezing in liquid nitrogen and subsequent thawing. The haemolysates were centrifuged at 40 000 *g* for 10 min. One part of the supernatant was filtered through a G25 Sephadex column at a flow rate of 15 drops min^{-1}. Eluates of carp and human samples in 1 mmol l^{-1} Tris-HCl buffer at pH 8·3 and pH 8·0, respectively (supplemented with 100 mmol l^{-1} NaCl and 1 mmol l^{-1} EDTA), were sampled automatically in separate glasses every 40 drops. Radioactivity and haemoglobin concentration were determined in all

samples as described before. Gel filtration was carried out immediately after the labelling procedure in haemolysates of carp and man. In carp, gel filtration was also performed 35 days after reinjection of labelled cells.

Another part of the carp haemolysate was diluted with 5 mmol l^{-1} Tris-HCl buffer pH 8·3, to give a final concentration of approx. 0·02 g l^{-1} haemoglobin. To avoid autoxidation, this solution was flushed with carbon monoxide for 10 min. Thin-layer polyacrylamide gel electrofocusing (Drysdale *et al.* 1971; Albers *et al.* 1981; Albers, 1985) was done with commercially available Servalyt Precotes pH 5–8, 125 mm × 125 mm × 0·15 mm, T5 C3 (Serva, Heidelberg, FRG). With a distance between the anode and the cathode of 100 mm and electrode solutions of 40 mmol l^{-1} DL-glutamic acid (anode), 0·1 mol l^{-1} NaOH (cathode), sample papers containing 5 μl of pretreated haemolysate were placed 30 mm from the cathodal contact strip. Temperature was 4°C (Haake T52 thermostat), pre-focusing time was 1 h and sample running time was 6 h using an LKB 2117 Multiphor II electrophoresis unit (LKB, Gräfelfing, FRG). An LKB 2103 power supply was set at a constant 1·5 W, maximum voltage of 2 kV and maximum current of 8 mA. Detection was by staining with Coomassie Blue R-250 (Serva Blue-G); ^{14}C radioactivity on the electropherogram was scanned with an Isomess (Isotopenmeßgeräte, Straubenhardt, FRG) and autoradiography of the electropherogram was done with X-ray screen blue base film and Röntgenschnell-Kodak developer.

To test whether ^{14}C was present in haem as well as in the protein fraction of haemoglobin, the following procedure was used. Part of the haemolysate of carp blood taken 35 days after labelling was dialysed against distilled water. After ultrafiltration, a 20:1 (v/v) excess of 0·2 mol l^{-1} HCl/acetone was added drop by drop at −20°C. The solution was kept at −20°C for 10 min with occasional stirring. The precipitate was washed in distilled water (Rossi Fanelli *et al.* 1958). The method of Lowry *et al.* (1951) was employed for determination of protein concentration in this solution. The filtrate was analysed by photometry at wavelengths from 400 nm to 650 nm using an LKB Ultra Spec 4050 (LKB, Gräfelfing, FRG) to identify the chlorhaemin. ^{14}C radioactivity was measured in both the globin and the haemin solution.

Statistical tests

Two-way analysis of variance including the *F*-test, the standard error of fraction means (S.E.M.), Fisher's least significant difference (LSD), and the multiple *t*-test (Holm, 1979) was used for all comparisons of fraction means (Sachs, 1984). The levels of significance calculated by algorithm 8.9 of Cooke *et al.* (1982) are shown in the tables. In addition, multiple regression (analysis of covariance) was calculated by standard procedures.

Results

Density, MCV, MCH and MCHC in red cell fractions

Densities of red cells of fractionated and unfractionated carp and human blood

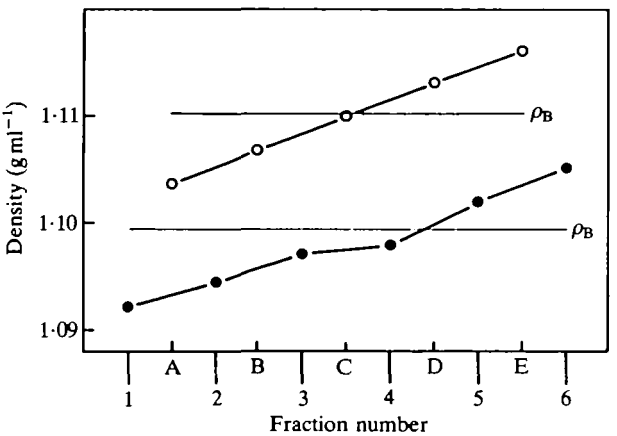

Fig. 1. Average densities of erythrocytes in five equal-sized fractions (A–E; top–bottom) of carp blood (○, $N = 6$) and in six equal-sized fractions (1–6; top–bottom) of human blood (●, $N = 5$). Lines (ρ_B) indicate average densities of non-fractionated cells.

are shown in Fig. 1. The linear gradients obtained from both species indicate that the population of red cells consists of subpopulations with different specific gravity. The two-way analysis of variance yielded a standard error of fraction means of ±0·0012. The least significant difference for $P < 0{\cdot}05$ was 0·0027. All adjacent pairs of points in Fig. 1 are significantly different, except for fractions 3 and 4 in human red cells. Haematocrit and haemoglobin concentration in the resuspended fractions of erythrocytes provided arbitrary data only; the resulting quotients for MCV, MCH and MCHC were suitable for haematological characterization. For carp, MCV, MCH and MCHC values in fractions increased from top to bottom, whereas in human erythrocyte fractions MCV decreased, MCHC increased and MCH remained constant from top to bottom (Fig. 2; Table 1). The mean values for whole blood of carp ($N = 45$) are also given in Table 1. The correlations between density and the haematological parameters in Table 1 were highly significant. The result of the Holm test was identical with the least significant difference test for adjacent pairs.

Scanning stage absorbance cytophotometry

Microscope photometry at the Soret wavelength (411 nm) allows the cells to be classified according to size (projected area), shape and absorbance (mean local absorbance and total integrated absorbance). Categorization of immature erythrocytes was done by visual screening of fractionated red cell populations. Such stages, circular in outline, were abundant in the light red fraction on top of the packed columns (21 % of the erythrocytes), whereas in the next two fractions only 5% and 1·5%, respectively, of all red cells could be identified as circular immature forms. In the last three fractions such forms could not be detected. All

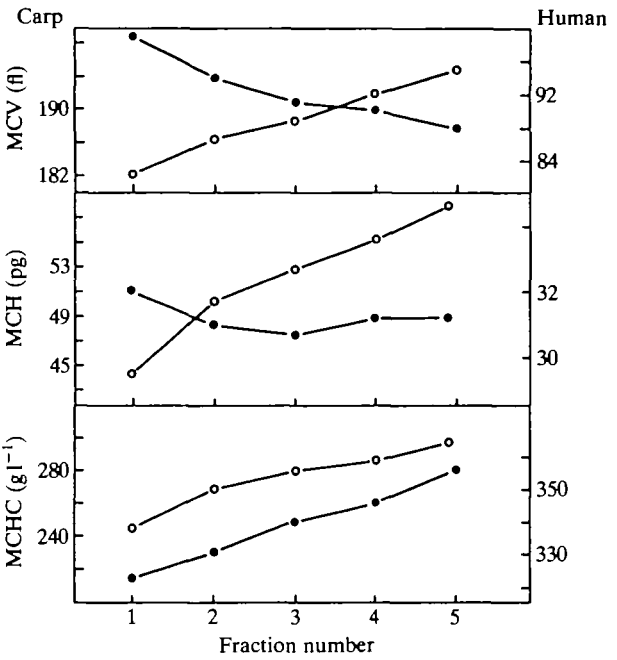

Fig. 2. Comparison of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) of fractionated carp ($N = 10$) and human ($N = 5$) blood cells (fractions 1–5; top–bottom).

other fractions contained almost exclusively cells that were slightly elliptical to elongated.

The projected area of the cells varied considerably within each fraction, as is obvious from the standard error given in Table 2. Part of this variation was due to shrinkage during the preparation of the slides. This source of error, however, could not have influenced the total integrated absorbance, which increased continuously from light to heavy fractions. Corresponding values for human red cells remained virtually constant (Table 2; Fig. 3). Accordingly, the histograms of the integrated total haemoglobin absorbances of density-separated carp erythrocytes showed a modal right-shift with fractions of increasing density. A typical distribution profile for carp is shown in Fig. 4. The distribution profiles in the two other specimens displayed the same pattern. In the top fraction the lowest haemoglobin content was 12–18 units and the highest 54–60 units. In the bottom fraction the lowest class was 48–54 units and the highest 84–90 units. Thus, low haemoglobin contents were only found in the lighter fractions of erythrocytes. In contrast, the density fractions of human red cells showed a nearly identical distribution of haemoglobin content in all fractions with a maximal absorbance in the class 34–40 units. In healthy humans the mean haemoglobin content of red cells is of the order of 30–32 pg. This allows a rough calibration of the absorbance data.

Isotope labelling in vitro

[2-^{14}C]glycine was incorporated into mature red blood cells of both carp and man during incubation *in vitro*. In five density fractions of a human blood sample pulse-labelled for 10 h and analysed after 26 h in unlabelled plasma, liquid scintillation counts were almost equally distributed and similar to the plasma value. In contrast, after extracorporal pulse-labelling of carp ($N = 5$) blood, reinjection and examination 2 days later, the intraerythrocytic radioactivity even in the bottom fractions was about 15 times greater than the plasma value. In addition, decreasing values were obtained in fractions of increasing density (Table 3).

For identification of ^{14}C-labelled soluble constituents in erythrocytes, haemolysates were prepared from carp and human blood immediately after labelling with [2-^{14}C]glycine *in vitro*. After gel filtration, radioactivity and haemoglobin concentrations were estimated in numerous fractions of the eluate. From human samples, ^{14}C-labelled substances were mainly retrieved as low M_r solutes, probably glycine (Fig. 5A). In carp haemolysates, considerable radioactivity was found in fractions of the eluate rich in haemoglobin (Fig. 5B). That the first peak in Fig. 5B was haemoglobin was confirmed by its spectral properties and by the fact that

Table 1. *Haematological characterization of density-separated carp erythrocytes; mean values from 10 specimens*

	MCV (fl)	MCH (pg)	MCHC ($g l^{-1}$)
Fraction			
1	182·1	44·6	244·7
2	186·4	50·2	268·9
3	188·6	52·9	280·0
4	192·0	55·1	286·7
5	194·9	58·0	297·6
P	<0·0001	<0·0001	<0·0001
S.E.M.	±1·55	±0·86	±4·42
LSD	3·70	2·04	10·55
r	0·73	0·88	0·81
Whole blood*	182·2 ± 19·9	50·5 ± 6·1	278·6 ± 23·9

Fraction numbers; 1 is the top fraction, 5 the bottom fraction.
MCV, mean corpuscular volume.
MCH, mean corpuscular haemoglobin content.
MCHC, mean corpuscular haemoglobin concentration.
P is the level of significance from *F*-test.
S.E.M. is the standard error of fraction means from two-way analysis of variance.
LSD is the least significant difference at the 5% level.
r is the multiple correlation coefficient significant with $P < 0{\cdot}0001$.
* Mean value and standard error ($N = 45$ carp).

Table 2. *Haemoglobin content of individual erythrocytes of density-separated carp blood; mean values for 100 cells*

Fraction	Haemoglobin absorbance*		Projected area (μm^2)	
1	36·6		62·5	
2	45·19	$P < 0{\cdot}0001$	69·0	$P < 0{\cdot}025$
3	50·97	S.E.M. ± 1·65	69·8	S.E.M. = ±1·82
4	55·15	LSD = 4·23	71·2	LSD = 4·66
5	59·01	$r = 0{\cdot}95$	73·4	
6	62·76		73·3	

* Integrated absorbance at 411 nm of one cell (arbitrary units; one unit corresponds roughly to 0·8 pg of haemoglobin).

The projected area is for one cell.

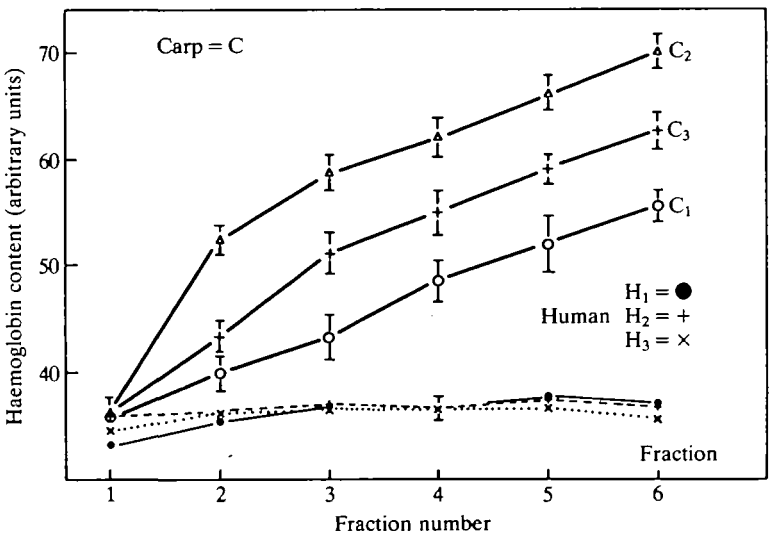

Fig. 3. Mean haemoglobin content of individual erythrocytes in carp and human blood ($N = 3$) obtained by cytophotometry; bars indicate standard deviation of 100 randomly chosen cells (1 = top fraction; 6 = bottom fraction).

no other substance present in red cell haemolysates can react with $K_3Fe(CN)_6$ and KCN to form cyanmethaemoglobin.

Isoelectric focusing of radiolabelled carp haemolysates is shown in Fig. 6A. Carp haemoglobin consists of three major components (Gillen & Riggs, 1972; Weber & Lykkeboe, 1978; Albers *et al.* 1981). In Fig. 6 these components show subcomponents, possibly due to the formation of hybridization products. The red colour in all the components in Fig. 6 showed that they were haemoglobin. It was shown by autoradiography (Fig. 6B) that all these components displayed ^{14}C radioactivity.

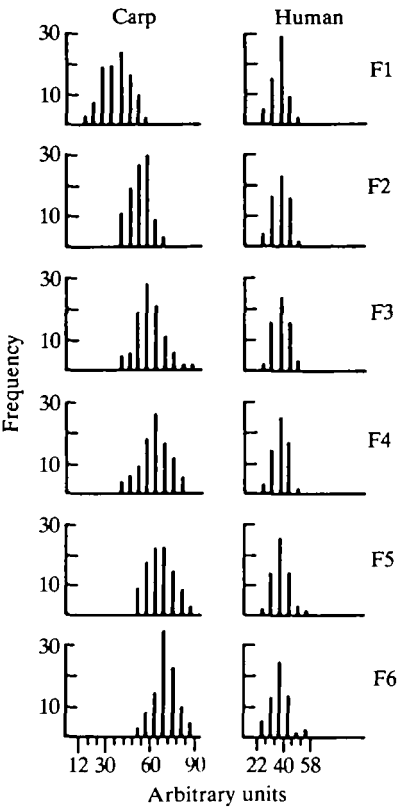

Fig. 4. Typical frequency distribution of total haemoglobin absorbances in individual carp and human erythrocytes obtained by cytophotometry (F1 = top fraction; F6 = bottom fraction).

Table 3. *Percentage distribution of radioactivity of density-separated erythrocytes, 2 days after labelling with [2-^{14}C]glycine*

Fraction	Carp blood ($N = 5$)	Human blood ($N = 1$)
1	46·1	21·3
2	20·2	20·1
3	13·8	19·8
4	11·3	19·7
5	8·6	19·1
χ^2*	46·27	0·13
P	6×10^{-7}	NS

Values for carp blood are means of five specimens.
* χ^2-test showing highly significant deviation from even distribution (for carp blood).

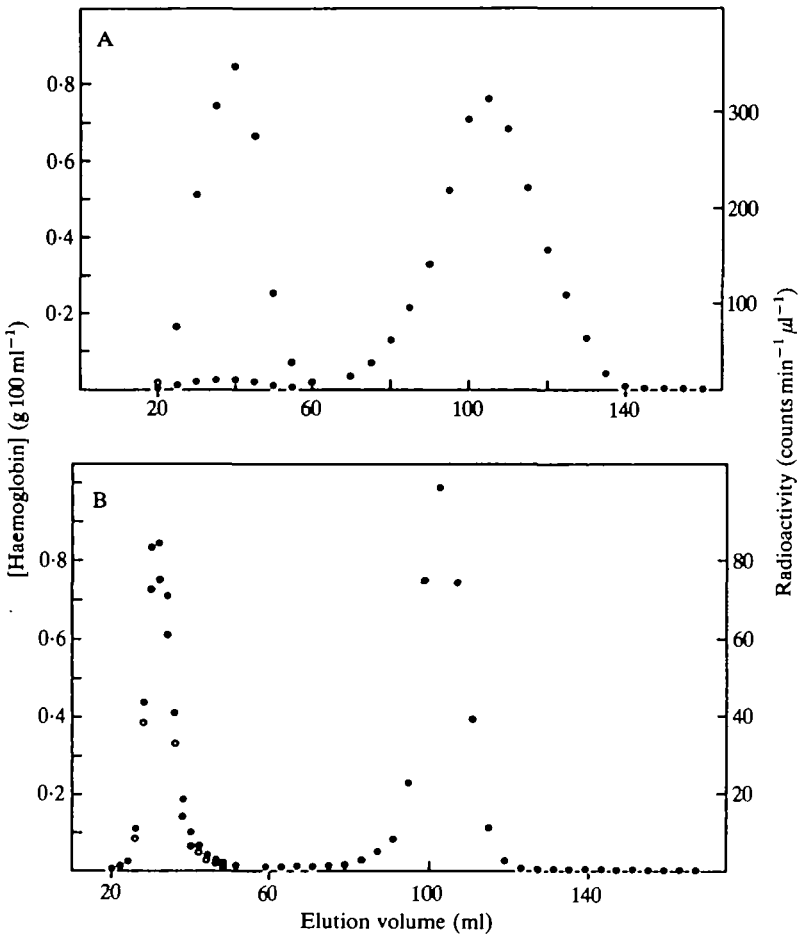

Fig. 5. Distribution of haemoglobin concentration (○) and ^{14}C scintillation counts (●) in the eluate of gel filtration obtained from (A) human and (B) carp haemolysates after incubation with [2-^{14}C]glycine *in vitro*.

Isotope labelling in vivo

Extracorporal labelling of carp erythrocytes with [2-^{14}C]glycine, and subsequent reinjection of the washed and resuspended cells, provided accurate isotopic dating of this cell type; direct injection of the marker would distribute the glycine widely in the amino acid pool. The mean values obtained in five specimens are summarized in Fig. 7. After 7 days, more than 60 % of the radioactivity was found in the two top fractions and only 7·3 % in the bottom fraction. With time, the radioactivity decreased in the top fraction and increased in the heavier fractions. The pattern of distribution of radioactivity was reversed within about 3 months. However, radioactivity was still present in the light fractions even after 203 days, indicating reutilization of [2-^{14}C]glycine.

Gel filtration of a sample on day 35 showed that the bulk of the scintillation

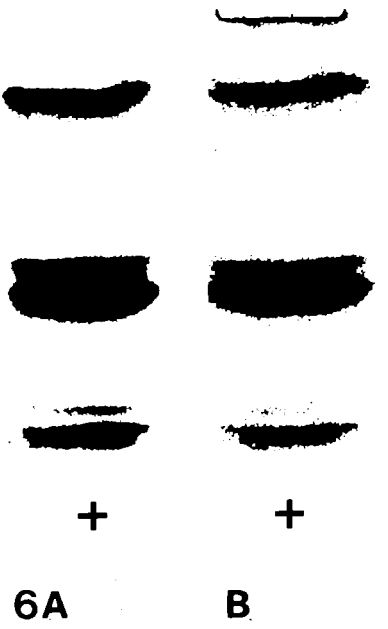

Fig. 6. (A) Analytical thin-layer electrofocusing of radiolabelled carp haemolysate; (B) autoradiograph of A.

counts co-eluted with haemoglobin (Fig. 8). In addition, separation of haemoglobin into the haem and globin moieties revealed that [2-^{14}C]glycine was incorporated into both components. The ratio of scintillation counts in haemin and globin was approximately 1:2. In two experiments the percentages of radioactivity in haemin were 35·7 and 31·7 %. Chlorhaemin (haem in acetone/HCl) was identified by its absorption spectrum in the visible range. Correspondingly, 64·3 % and 68·3 % of the radioactivity was found in the globin fraction.

Discussion

Centrifugation is a proved means of obtaining density separation in non-nucleated erythrocyte populations (Hoffman, 1958; Prankerd, 1958; Danon & Marikovsky, 1964). Effective separations can be obtained from ultracentrifugation in swinging bucket rotors (Rigas & Koler, 1961; Garby & Hjelm, 1963; Piomelli *et al.* 1967) and further improvements can be achieved with angle-head rotors (Murphy, 1973) which allow rotation of the cells within the tubes, resulting in a very effective packed cell separation (Wolowyk, 1982). Cohen *et al.* (1976) stressed the numerous advantages of Murphy's method which was successfully used by Trautsch *et al.* (1981), Fischbeck *et al.* (1982), Grzelinska *et al.* (1983) and Bartosz *et al.* (1987). A multiple-step Percoll gradient would possibly yield better-defined density classes. However, since we found difficulties with some biochemical tests,

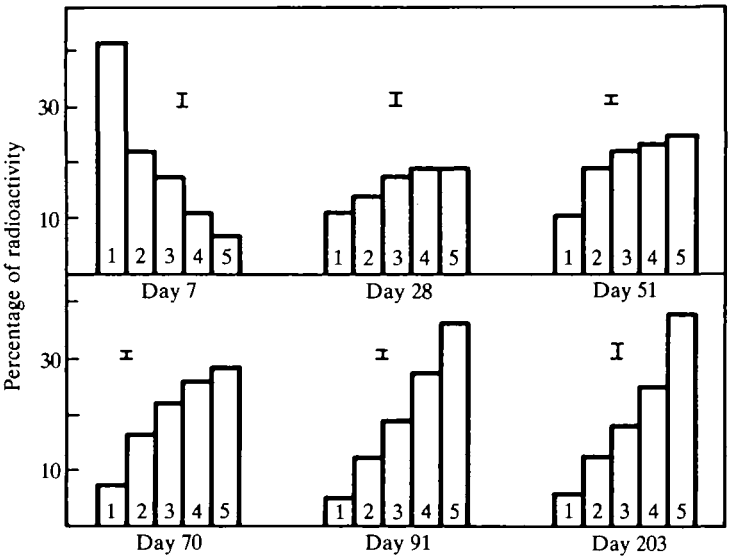

Fig. 7. Percentage of radioactivity in fractions 1 (top) to 5 (bottom) in ^{14}C-labelled carp erythrocytes *in vivo* from 7 to 203 days after labelling with [2-^{14}C]glycine. Mean values of five specimens. Bars indicate standard error of fraction means from two-way analysis of variance.

such as determinations of enzyme activities, in Percoll-separated erythrocytes, we preferred Murphy's method. Lane *et al.* (1982) suggested velocity sedimentation at unit gravity in a buffered bovine serum albumin gradient for separating trout

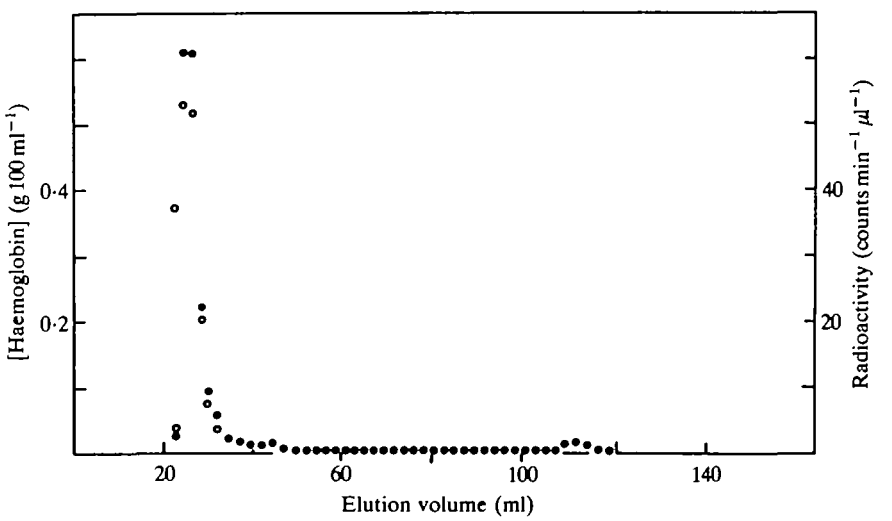

Fig. 8. Distribution of haemoglobin concentration (○) and ^{14}C-scintillation counts (●) in the gel filtration eluate obtained from a haemolysate of carp blood taken 35 days after pulse-labelling with [2-^{14}C]glycine.

(*Salmo gairdneri*) erythrocytes. This method gave the lowest values of MCHC in the bottom and top fractions and higher values in the intermediate fractions. Since all physical evidence suggests a positive correlation between density and MCHC, we felt this method would not be suitable for our purpose. The efficiency of our method was confirmed by the results of separation with phthalate ester gradients, as well as by the results in human erythrocytes which confirmed the well-established findings (Prentice & Bishop, 1965; Piomelli *et al.* 1967; Cohen *et al.* 1976).

A correlation between density and age has been established for non-nucleated erythrocytes by various methods involving isotopic markers (i.e. Prentice & Bishop, 1965; Bishop & Prentice, 1966; Danon *et al.* 1966; Piomelli *et al.* 1967). An analogous density–age relationship based on morphological data has been proposed for trout erythrocytes (Lane *et al.* 1982). However, the efficiency of the fractionation procedure and the correlation between age and density of red cells remains open to discussion (Beutler, 1985, 1986; Piomelli *et al.* 1986).

The same conclusion follows for carp erythrocytes from our experiments with ^{14}C-labelled glycine. Even if some reutilization of glycine were to occur, the fractional distribution of radioactivity and the inversion of the histogram (Fig. 7) within 200 days strongly support a correlation between age and density. Similarly, the modal increments in the distribution profiles of the haemoglobin content in individual erythrocytes (Fig. 4) provide evidence for such a correlation. These arguments are limited by the possibility of a fractional inhomogeneity with age. However, from gross morphological examinations and the nearly symmetrical frequency distributions (Fig. 4) it can be concluded that the density fractions were reasonably homogeneous.

The differences between mammalian and fish erythrocytes seem to be of importance (Fig. 2): mammalian red cells shrink in volume during ageing but retain a constant haemoglobin content. Thus, the increase in haemoglobin concentration, and therefore in density, is due to a reduction in volume. In contrast, during ageing, carp red cells increase not only in haemoglobin content but also in volume. An increase in volume in other cyprinid red cells has been reported by Weinberg *et al.* (1973) and Lane *et al.* (1982). Thus, the increases in haemoglobin concentration and density result from the haemoglobin content rising more than the volume. Erythrocytes can only increase their haemoglobin content by synthesis. From the continuous rise in haemoglobin content it is obvious that fish erythrocytes must synthesize haemoglobin after being released into the circulation.

This finding is in line with cytophotometric investigations of carp erythrocyte populations under conditions of respiratory stress (Schindler & de Vries, 1986). However, it would appear from fine morphological data that mature erythrocytes lack the capacity to synthesize haemoglobin. There is a well-defined sequential correlation between the appearance of mitochondria and polyribosomes and the accumulation of haemoglobin (Sekhon & Beams, 1969; Yamamoto & Iuchi, 1976). However, polyribosomes, abundant in immature red cells, diminish in number or

seem to vanish during maturation, as do other organelles obligatory in biochemical syntheses (Sekhon & Beams, 1969; Yamamoto & Iuchi, 1976; Lane & Tharp, 1980; Lane *et al.* 1982). Such observations, however, are somewhat difficult to evaluate since ribosomes are hardly discernible from the rather dense backgrounds in cells with a very high concentration of haemoglobin. Our own, as yet unpublished, fixed morphological studies, including freeze–fracture techniques, show that all carp erythrocytes possess numerous pores in the nuclear envelope and contain several variform vacuolar compartments within the cytosol with at least some endocytic activity present on the plasma membrane. Moreover, nucleoside triphosphate contents increased continuously with ageing of red cells of rainbow trout (Lane, 1984). Taking all arguments together, the morphological evidence is not decisive about the ability of circulating erythrocytes to synthesize haemoglobin. A biochemical approach, however, seems to favour this hypothesis.

Incubations with [2-^{14}C]glycine gave information about haemoglobin synthesis *in vitro*. This amino acid marker can label the globin component intratranslationally and is utilized in haem synthesis (Hevesy *et al.* 1964; Piomelli *et al.* 1967). Both carp and human erythrocytes took up [2-^{14}C]glycine. Whereas the former incorporated the labelled amino acid into haemoglobin, the latter merely absorbed it (Fig. 5; Table 3). Radioactivity was present in haemoglobin-containing fractions of carp but not human gel filtrates of haemolysates. After cleavage of carp haemoglobin, ^{14}C radioactivity was detected in both the globin and the haem component. Autoradiographic evidence suggests incorporation of [2-^{14}]glycine into all three major components of carp haemoglobin.

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